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Ex Vivo Evaluation of Reduced Myocardial ß-Adrenergic Receptor Binding in the Streptozotocin-Treated Hyperglycaemic Rats Using (S)- [^{3}H]CGP12177

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Ex Vivo Evaluation of Reduced Myocardial β-Adrenergic Receptor Binding in the Streptozotocin-Treated Hyperglycaemic Rats Using (S)-[^{3}H]CGP12177

BY

MARYAM PARSA-NEZHAD

This Thesis is Submitted as a Partial Fulfillment of the Master of Science Program in Cellular and Molecular Medicine

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Ex Vivo Evaluation of Reduced Myocardial β-Adrenergic Receptor Binding in the Streptozotocin-Treated Hyperglycaemic Rats Using (S)-[^{3}H]CGP12177

MSc Thesis 2008, Maryam Parsa-Nezhad

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Type II Diabetes Mellitus (DM) is a common metabolic disorder that affects millions of people worldwide. Elevated blood glucose levels in type II DM is associated with altered sympathetic nervous system (SNS) activity and enhanced noradrenaline (NA). Persistent NA release, in turn, leads to alterations in β-adrenergic receptor (βAR) density and downstream signalling. A common complication of diabetes is cardiovascular disease. In this project, myocardial βAR density was evaluated in hyperglycaemic streptozotocin (STZ)-treated rats fed high fat diet (HFD), using βAR antagonist (S)-[^{3}H]CGP12177. At 30 minutes post injection, the radiotracer exhibited specific binding in myocardial regions, brown adipose tissue and kidney, and was capable of measuring reduced binding to βARs. A subset of HFD moderate-STZ treated rats became hyperglycaemia, whereas the remainder maintained euglycaemia. At 10 days post-STZ, no alteration was observed in tracer binding in either sub-group. At 56 days, hyperglycaemic rats displayed a significant reduction in specific binding to βARs in myocardial regions (30-40%), while no alteration was observed in euglycaemics or controls. This finding suggests a strong association between sustained hyperglycaemia and alterations in SNS activity and βAR binding.

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^{11}C	carbon-11
[^{11}C]CGP12177	[^{11}C] 4-(3-butylamino-2-hydroxy-propoxy)-benzimidizol-2-one
[^{11}C]CGP12388	[^{11}C]4-(3-(2-isopropylamino)-2-hydroxy-propoxy)-2-H-benzimidazol-2-one
[^{11}C] HED	[^{11}C] hydroxyephedrine
[^{11}C]MIBG	*meta*-iodobenzylguanidine
^{3}H	tritium
[^{3}H]CGP12177	[^{3}H] 4-(3-butylamino-2-hydroxy-propoxy)-benzimidizol-2-one
[^{3}H]DHA	[^{3}H]dihydroalprenolol
^{125}I	iodine
[^{125}I]CYP	[^{125}I]cyanopindolol
[^{125}I]ICYP	[^{125}I]iodocyanopindolol
[^{125}I]IHYP	[^{125}I]iodohydroxybenzylpindolol
AC	adenylyl cyclase
Ad	adrenaline
ANS	autonomic nervous system
ATP	adenosine triphosphate
βAR	β-adrenergic receptors
βARK	β-adrenergic receptors kinase (or GRK)
B_{max}	maximal binding density
cAMP	3, 5 adenosine monophosphate
CHF	conjestive heart failure
cpm	counts per minute
CVD	cardiovascular diseases
DAN	diabetic autonomic neuropathy
DM	diabetes
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acid
GDP	guanosine diphosphate
GLUT-2	glucose transporter 2
GLUT-4	glucose transporter 4
GPCR	G protein coupled receptor
GTP	guanosine triphosphate
HSL	hormone sensitive lipase
IGT	impaired glucose tolerance
IR	insulin receptor
K_d	dissociation constant
LS	liquid scintillation
NA	noradrenaline
NAT-1	noradrenaline transporter-1

PET	positron emission tomography
PI-3K	phosphoinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B(Akt)
PKC	protein kinase C
PMT	photomultiplier tube
PPARγ	peroxizome proliferators–activated receptor-γ
pSNS	parasympathetic nervous system
SNS	sympathetic nervous system
SR	sarcoplasmic reticulum
STZ	streptozotocin
TZD	thiazolidinedions
UCP-1	uncoupling protein 1

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To begin with, now that my thesis work is complete, I would like to thank everyone who assisted me in the completion of this very important goal. Firstly, I would like to acknowledge my Supervisor, Dr. Jean DaSilva. He kindly taught me the principles of research and patiently guided me through my project over the last two years. I truly appreciate all his dedication and effort in helping me take my first steps into the world of research. Secondly, I would like to thank all my colleagues and friends: Miran, James, Michael, Sheryn, Tayebeh, Jeff, Steph, Sam, and Paul. As all my colleagues well know, no lab work is successfully performed without the strong collaborative efforts of those around you. Without these people, my project would not be complete today. I would like to give special thanks to Miran who assisted to write this manuscript and to James who helped me out in every step of the way.

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Thank you all!

1.0 INTRODUCTION

1.1 General Introduction

Diabetes mellitus (DM) is a common metabolic disorder worldwide and affects as many as 2.4 million Canadians. DM is classified into type I and type II, of which the latter accounts for approximately 90% of DM diagnosis (Shehadeh and Regan, 1995; Stumvoll et al., 2005). The presence of DM is associated with high incidence of cardiovascular diseases (CVD) (Gaede et al., 2003; Wilson et al., 2005). Furthermore, CVD, and congestive heart failure (CHF) are associated with an approximately 25% increased risk of developing metabolic abnormalities and subsequent diabetes in comparison to 7% in the general population (Paolisso et al., 1991; Swan et al., 1997; Kostis and Sanders, 2005). CVD, CHF, and DM are associated with altered sympathetic nervous system activity (Pagani et al., 1988; Bellavere et al., 1992; Brodde, 1993; Brodde and Michel, 1999; Manzella et al., 2001; Brouri et al., 2002; Caballero, 2005).

1.2 Sympathetic Nervous System Activity

1.2.1 Noradrenaline Release

The autonomic nervous system (ANS) innervates the majority of the organs of the body and regulates important physiological functions including cardiac control and energy metabolism (Nonogaki, 2000; Manzella and Paolisso, 2005). The ANS is divided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (pSNS), both containing preganglionic neurons that originate in the central nervous system and synapse with postganglionic neurons in the peripheral ganglia (Figure 1.1).

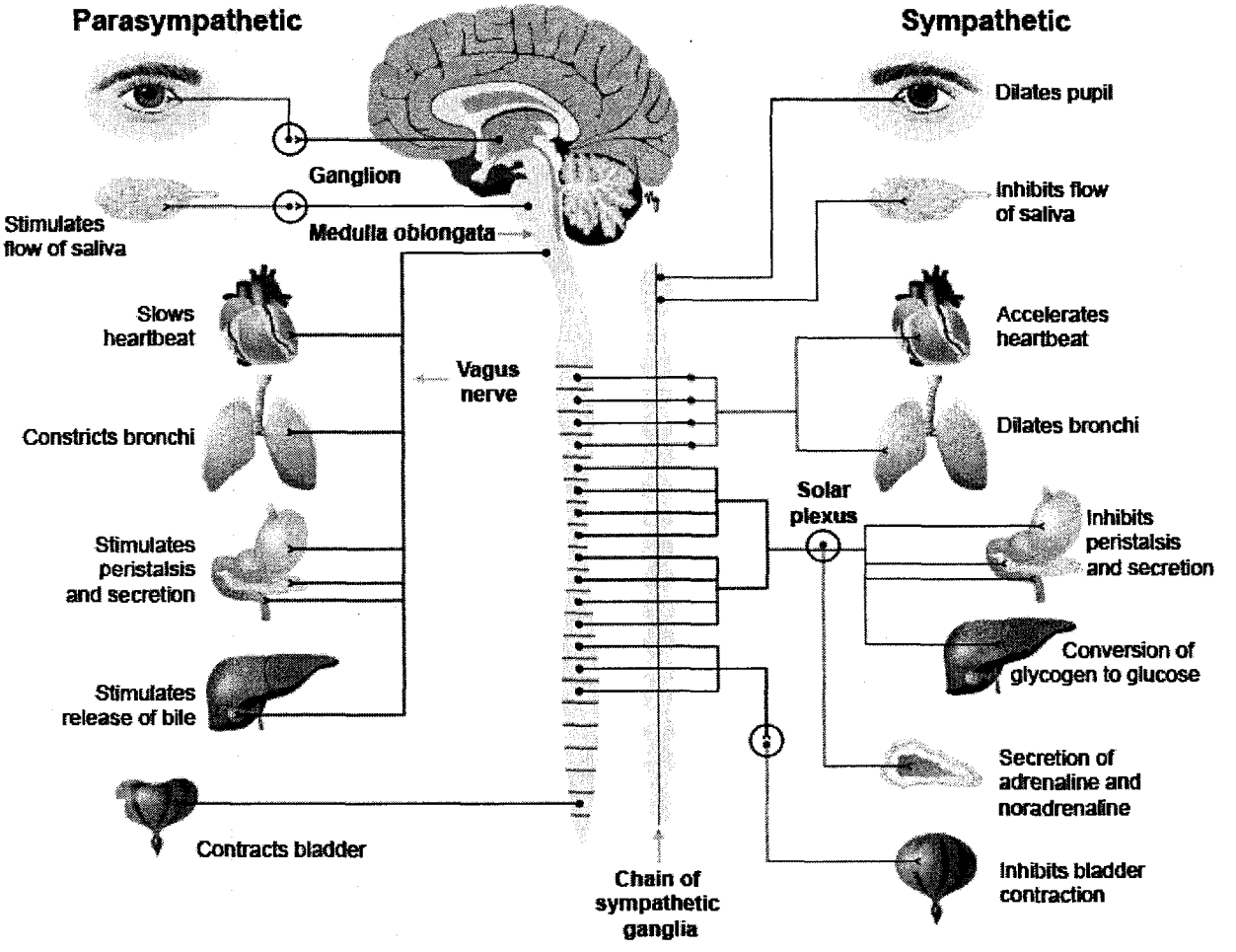


Figure 1.1: Schematic illustration of autonomic nervous system which comprises two branches; sympathetic and parasympathetic nervous systems. They innervate target organs and mediate certain physiological pathways. Generally, the stimulatory effects of the SNS are antagonized by the pSNS. Adapted from www.users.rcn.com

Postganglionic neurons innervate the target organs and stimulate major metabolic pathways (Gardemann et al., 1992; Shimazu, 1996; Saxena et al., 1999; Romijn and Fliers, 2005). They also innervate the heart and stimulate heart rate, contractility, and cardiac output. The postganglionic fibres terminate in a bulbous swelling or bouton separated from the other neurons or the target organ by the synaptic cleft (Hoffman and Lefkowitz, 1995) (Figure 1.2). The signal transduction is mediated through release of catecholamines from postganglionic nerve terminals to the synaptic area. PSNS releases acetylcholine, acting on cholinergic muscarinic receptors, and SNS releases noradrenaline (NA) to the synaptic area in the heart. NA is synthesized from tyrosine in a multi-step reaction and is stored in vesicles. Upon arrival of the signal, vesicles are infused with plasma membrane and NA is released. NA then binds to adrenergic receptors and stimulates specific intracellular pathways. There are two main classes of adrenergic receptors, α and β. These are further subdivided into several subtypes; α_1, α_2, β_1, β_2, and β_3 (Lafontan and Berlan, 1993; Brodde et al., 2001). Adrenergic signal transduction is terminated by uptake of NA from the synaptic cleft through noradrenaline reuptake-1 (NAT-1) or by metabolism of NA by specific enzymes (catechol-*O*-methyl transferase and mitochondrial mono-amine oxidase) (Kopin and Gordon, 1963; Lowe et al., 1975; Raffel et al., 1996). In general, the stimulatory actions of the SNS are antagonized by the pSNS.

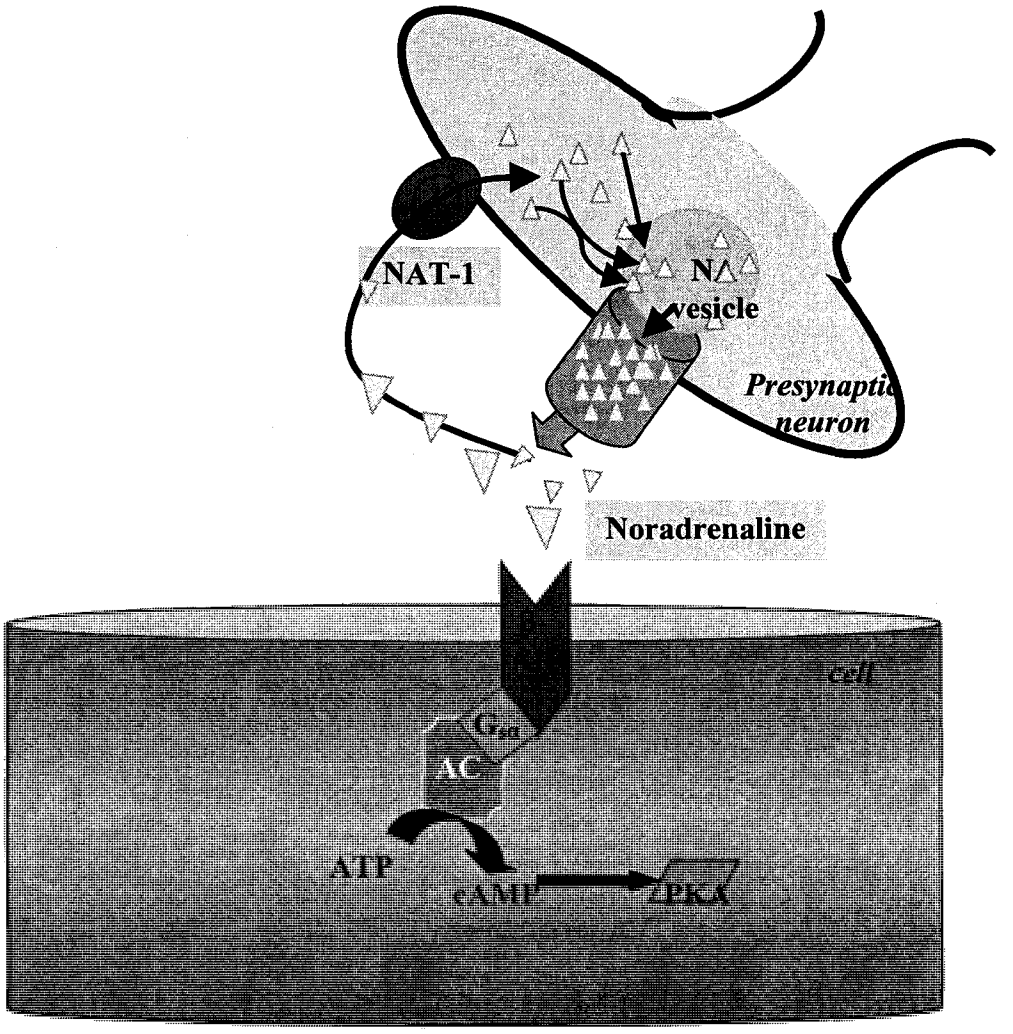


Figure 1.2: Schematic illustration of synaptic cleft. Upon arrival of the signal, NA vesicles release NA to synaptic areas. NA then binds to the receptors (βARs) and initiates the intracellular signal transduction. Signalling is terminated via reuptake of NA from synaptic area through NAT-1. Adapted from Yoshinaga et al (2005), Current Pharmaceutical Design, 7:903-32

1.2.2 G Protein Coupled Receptors

Adrenergic receptors belong to the superfamily of G protein coupled receptors (GPCRs). Post-synaptic cell signalling is mediated by GPCRs following stimulation of neurotransmitters, hormones, or growth factors (Strader et al., 1994). Despite the wide variety of functions, GPCRs, including βARs, exhibit homology in the structure with the common 7 transmembrane α-helical structure linked by 3 intracellular and 3 extracellular loops with an extracellular amino terminus and intracellular carboxy terminus (Figure1.3) (Pierce et al., 2002; Preininger and Hamm, 2004). GPCRs have an extensive interaction with heterotrimeric G proteins. G proteins are composed of 3 subunits, α, β, and γ. Multiple types of G proteins including G_s, G_i, G_o and G_q have been identified to date, each performing a certain physiological role in different cell types (Dzimiri, 1999). Among these proteins, G_s and G_i are coupled to different subtypes of βARs and modulate the intracellular signalling processes that regulate cardiac function or energy expenditure (Feldman, 1993; Daaka et al., 1997; Lefkowitz, 1998; Dzimiri, 1999; Brodde et al., 2006).

1.2.3 β-Adrenergic Signalling

βAR signalling is modulated mainly via the G_s pathway (Figure 1.4). In the G_s pathway, the G_α subunit plays an integral role in mediating the message and commencing the intracellular signalling (Neves et al., 2002). G_α possesses GTPase intrinsic activity and therefore has the potential to exchange guanine nucleotides, GDP and GTP, under certain conditions (Spiegel and Weinstein, 2004). In the basal inactive state, GDP is bound to the catalytic site of GTPase on the G_α subunit.

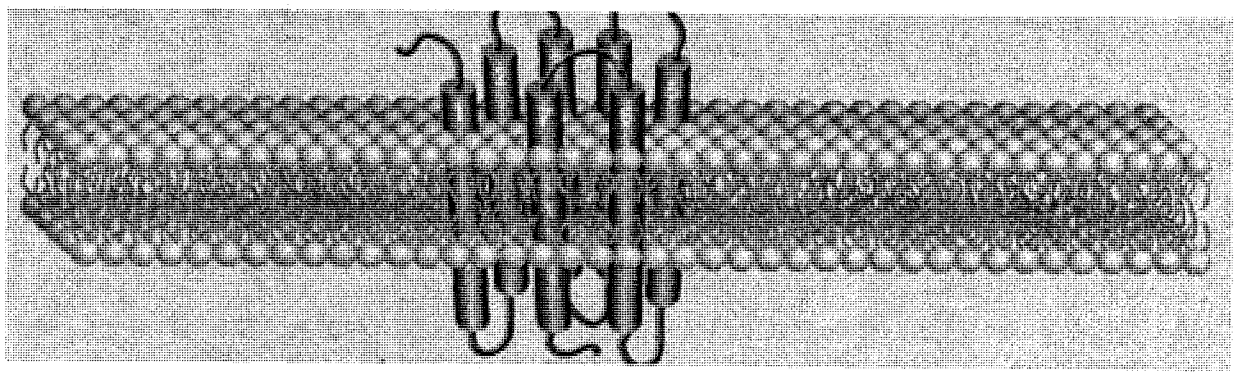


Figure 1.3: Schematic representation of 7 transmembrane α-helical structure of the GPCRs including βARs with 3 intracellular and 3 extracellular loops. (amino terminus is extracellular and carboxy terminus is intracellular). Adapted from Pao et al (2002), Science's STKE:158-PE42

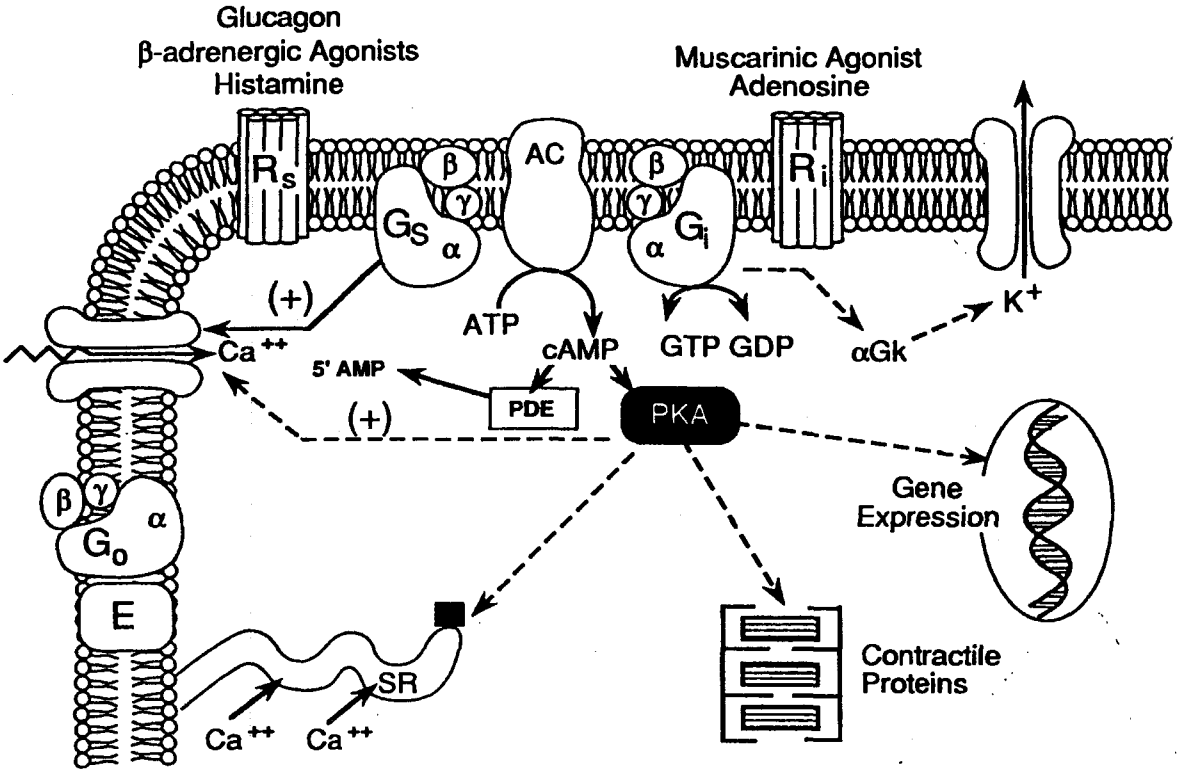


Figure 1.4: Schematic representation of βAR signalling. βAR is coupled to G_s, which, in the presence of GTP dissociates its α-subunit to stimulate AC. AC, in turn, mediates the production of cAMP and cAMP activates PKA. PKA phosphorylates a number of key regulatory proteins which are crucial for the ultimate response of the cells to adrenergic stimulation, including contractile responses of the heart. cAMP production and signalling pathway can be suppressed by activation of G_i pathway. Adapted from Feldman, M.D, Copelas, L et al (1987) Circulation, 75: 331-339

Upon stimulation by an agonist, βAR undergoes complex conformationals changes that facilitate G protein binding (Jansson et al., 1999). This process is known as activation that leads to exchange of GDP for GTP. Following binding of GTP, G_α dissociates from $G_{\beta\gamma}$ and activates adenylyl cyclase (AC) (Marinissen and Gutkind, 2001). This effector enzyme in turn mediates changes in the second messenger levels, cAMP (Hepler and Gilman, 1992), further activating a serine/theronine protein kinase known as protein kinase A (PKA). As discussed later, activation of PKA results in phosphorylation of certain target proteins. These key regulatory proteins eventually mediate a number of cellular functions such as metabolic regulation, muscle contraction, growth control and cell survival or death (Dzimiri, 1999; Marinissen and Gutkind, 2001; Karnik et al., 2003). β_2AR can also be coupled to G_i. Upon activation, G_i inhibits the current PKA pathway and stops further signalling (Broadley, 1999; Zheng et al., 2004; Brodde et al., 2006).

1.2.4 Localization and Functions of βARs

1.2.4.1 β_1AR

βARs are broadly distributed throughout the organs that are innervated by SNS. β_1AR is expressed in all parts of the heart and mediates the majority of responses to nonselective agonists (Brodde, 1991). Once stimulated, β_1AR triggers the cardiac contractile machinery that initiates with production of cAMP and activation of PKA and results in an increase in automaticity, conduction velocity (chronotropy), excitability and contraction force (inotropy) (Kaumann et al., 1989; Molenaar et al., 1997). β_1AR activates PKA through its interaction with G_s protein. PKA, in turn, mediates the phosphorylation of a number of Ca^{2+} handling proteins, each possessing a certain role in the regulation of Ca^{2+}

level and ultimately in the cardiac function (Figure 1.5). These regulatory proteins include sarcolemmal L-type Ca^{2+} channels, sarcoplasmic reticulum (SR), membrane protein and myofilament components (such as troponin 1 and C protein), phospholamban and ryanodine receptors (Lindemann et al., 1983; Insel and Ransnas, 1988; Schubert et al., 1989; Yatani and Brown, 1989; Fleming et al., 1992; Zheng et al., 2004).

1.2.4.2 β_2AR

β_2AR is mainly expressed in airway smooth muscle cells and mediates muscle relaxation and muscle tone in the lung (Nials et al., 1993; Taylor, 2006). In addition to its predominant role in the lung, β_2AR is also functionally expressed in human myocardium (Brodde et al., 1984; Bristow and Ginsburg, 1986; Bristow et al., 1986), as well as in rodents (Kitagawa et al., 1995; Horinouchi et al., 2006). The ratio of β_1 to β_2 is approximately 2:1 in the human heart (Bristow et al., 1986; Dzimiri, 1999). It is suggested that this ratio is as high as 4:1 in the rat left ventricle (Tseng et al., 2001). Table 1.1 displays the distribution of β_1AR and β_2AR is various rat myocardial regions. Similar to β_1AR, β_2AR contributes to contractile system of the heart through its interaction with G_s protein and activation of AC (Figure 1.5) (Waelbroeck et al., 1983; Brodde et al., 1984; Bristow et al., 1986). Contrary to β_1AR, β_2AR can also couple to G_i protein (Feldman, 1993; Xiao et al., 1995; Daaka et al., 1997; Brodde et al., 2006). Several lines of evidence indicate that G_i signalling is essential for the functional disruption of the cAMP signalling pathway in the heart. For instance, it has been shown that inhibition of G_i by pertusis toxin potentiates β_2AR-mediated positive inotropic effects in the rat heart (Xiao et al., 1995).

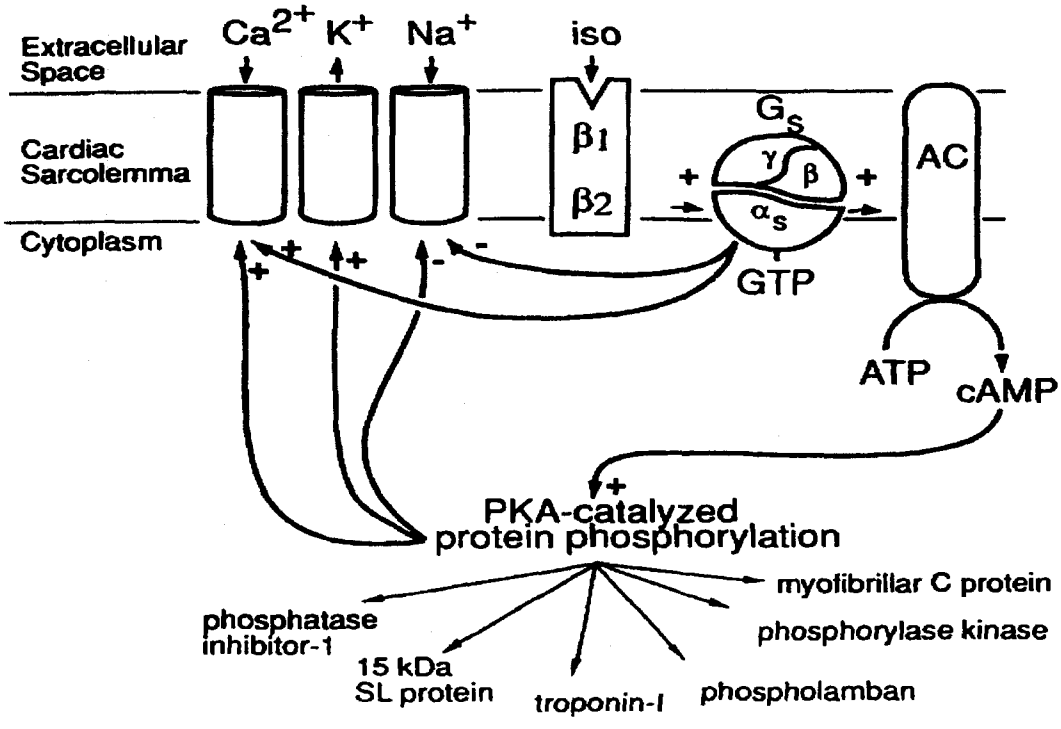


Figure 1.5: Schematic of βAR signalling in the heart. Both β_1- and β_2-AR are coupled to G_s, which upon stimulation dissociates its α-subunit to activate AC. This results in the formation of cAMP, which in turn, stimulate PKA to phosphorylate various cellular proteins involved in producing positive inotropic and chronotropic effects. Activated $G_{s\alpha}$ also appears to directly couple βAR to sarcolemmal Ca^{2+} channel for stimulation. Adapted from Fleming et al (1992), Circulation, 85:420-33

Table 1.1: Maximum binding density (B_{max}) of βARs in rat myocardial regions.

B_{max}(fmol/mg)	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum
β_1AR	48.2±6.2 [1]	50.4±7.5 [1]	35.9±0.6 [1]	34.0±3.6 [1]	37.9±5.9[1]
				24.6±1.2 [2]	
				21.7±2.0 [3]	
β_2AR	12.4±1.1 [1]	11.2±1.6 [1]	11.3±0.9 [1]	11.5±0.9 [1]	12.4±0.7[1]
				11.7±0.4 [2]	
				11.8±2.4 [3]	

References: 1: (Kompa et al., 1999), 2: (Sato et al., 1997), 3: (Sarsero and Molenaar, 1995). B_{max} is calculated as fmol/mg of protein.

It is believed that concurrent coupling of β_2AR to G_s and G_i and the counterregulatory actions of these two pathways aid to protect the myocytes against persistent stimulation of the receptors (Feldman, 1993; Ungerer et al., 1993; Dzimiri, 1999; Brodde et al., 2006). β_2AR is also present in the vasculature, mediating vasorelaxation through stimulation of the cAMP production (Gaballa et al., 1998).

Several studies have proposed novel roles for β_2AR in other tissues such as liver, skeletal muscle and pancreas. Studies on rat models of obesity and diabetes suggest the effect of the β_2-agonist, clenbuterol on enhanced glucose transport and decreased insulin resistance in the muscle (Castle et al., 2001; Pan et al., 2001). Furthermore, clenbuterol was found to induce insulin secretion in human islets incubated in a high dose of glucose (Lacey et al., 1990). Therefore, it is assumed that β_2ARs also have a role in the general glucose homeostasis.

1.2.4.3 β_3AR

β_3AR is thought to be expressed predominantly in adipocytes, mediating lipolysis and thermogenesis in white adipose and brown adipose tissues (Arch et al., 1984; Hollenga and Zaagsma, 1989; Collins and Surwit, 2001). The density of this βAR subtype in human adipocytes is clearly lower than that observed in rodents (Krief et al., 1993; Weyer et al., 1998). NA binding to βARs stimulates both G_s and G_i pathway, resulting in phosphorylation and subsequent activation of hormone-sensitive lipase (HSL) and periplipin A, further exposing the lipid droplet to HSL attack. Finally, HSL breaks the lipid droplet to triglycerides and free fatty acid (FFA) (Robidoux et al., 2004). Thermogenesis is mediated by βAR through initiating a series of events that ultimately results in upregulation of uncoupling protein 1 (UCP 1). UCP 1, in turn, allows the

leakage of protons across the inner mitochondrial membrane, dissipating the electrical gradient necessary for ATP generation (Argyropoulos and Harper, 2002).

In addition to adipocytes, β_3AR has also been localized in the heart, although its proportion compared to β_1AR and β_2AR is not estimated (Skeberdis, 2004). It is coupled to G_i-protein in ventricles, inducing negative inotropic effects, and protecting the heart in SNS overactivity conditions (Shen et al., 1994). Other tasks proposed for β_3AR include mediation of peripheral vasodilation mainly in the fat and skin (Berlan et al., 1994; Shen et al., 1994), and reduction of contractile activity in ileum and colon (Bond and Clarke, 1988; Manara and Bianchetti, 1990).

1.2.4.4 β_4AR

Evidence has recently suggested the existence of a fourth βAR in human atria (Molenaar et al., 1997). It has been shown that β_3AR-agonists suppress contraction in ventricles, while inducing positive inotropic effects in the atria. This suggests that inhibitory responses in ventricles are partly caused by stimulation of β_3AR, however, stimulatory responses in the atria may be mediated by another subtype (Gauthier et al., 1996; Skeberdis et al., 1997). The presence of a fourth βAR, that mediates stimulative effects of β_3AR-agonists in the atria has been speculated; although, more evidence needs to be accumulated on this subtype from cloning experiments (Dzimiri, 1999; Tseng et al., 2001). Table 1.2 exhibits total βAR distribution in different rat tissues.

Table 1.2: Maximum binding density (B_{max}) of βARs in different rat tissues. The methods used for determining βAR density include in vitro binding assay of various radiotracers in membrane preparations; the B_{max} is calculated as fmol/ mg of protein. References 1: (Kompa et al., 1999), 2: (Saraiva et al., 2003), 3:(Rothwell et al., 1985), 4: (Germack et al., 1997), 5: (Jensen et al., 2002), 6: (Snavely et al., 1982), 7: (Das et al., 2006).

Tissue	B_{max}(fmol/mg)	Radiotracer
Right Atrium[1]	60.5±6.1	(S)-[^{125}I]CYP
Left Atrium[1]	61.6±8.8	(S)-[^{125}I]CYP
Right Ventricle[1]	47.2±5.3	(S)-[^{125}I]CYP
Left Ventricle[1]	45.5±2.9	(S)-[^{125}I]CYP
Intraventricular Septum[1]	50.3±6.1	(S)-[^{125}I]CYP
Total Heart[2]	271±31.43	(S)-[^{3}H]CGP12177
Lung[3]	328±4	(S)-[^{3}H]DHA
Brown Adipose[3]	160	(S)-[^{3}H]DHA
White Adipose[4]	27±5	(S)-[^{3}H]CGP12177
Skeletal Muscle (soleus)[5]	32.8±0.9	(S)-[^{3}H]CGP12177
Kidney (cortex)[6]	46	(S)-[^{125}I]ICYP
Pancreas[7]	7.50±1.98	(S)-[^{3}H]Propranolol

1.2.5 βAR Regulation

GPCRs, including βARs, are synthesized in the golgi apparatus and delivered to the cell surface. Once βARs are stimulated by specific agonists, they trigger a cascade of events that ultimately leads to a specific response, such as contraction in myocytes. However, should signalling continue for longer periods, βARs undergo specific compensatory regulations including desensitization and downregulation.

1.2.5.1 Receptor Desensitization

Mechanisms involved in receptor desensitization mainly deal with short-term stimulation and rapid and reversible control of the receptor function. Desensitization is mainly characterized by a decrease in the ability of βARs to respond to agonist stimulation which leads to AC activation and cAMP production (Hadcock and Malbon, 1991). Three regulatory proteins have been found to participate in desensitization of the βARs; PKA, G protein receptor kinases (GRKs) (It is also termed as βAR kinase or βARK), and the arrestins (Lefkowitz, 1998). If the βAR stimulation and cAMP production persists for seconds to minutes, PKA phosphorylates the receptor through a feedback regulation. The PKA phosphorylation site is located in the third intracellular loop (Bouvier et al., 1988; Hausdorff et al., 1989; Brodde, 1993). It is believed that phosphorylation alters the conformation of the receptor, and thereby impairs its further interaction with G protein (Lefkowitz, 1993). This type of desensitization is not specific and any stimulant that elevates cAMP level has the potential to cause phosphorylation of any GPCR containing an appropriate PKA phosphorylation site (Lefkowitz, 1998). It is called heterologous or non-agonist-specific desensitization.

Homologous or agonist-specific desensitization includes two major steps. First, βARK phosphorylates exclusively the agonist-occupied receptors, then β-arrestin binds to the receptor, preventing its further reassociation to the G protein and thereby efficiently suppresses the signal transduction (Dzimiri, 1999; Miller and Lefkowitz, 2001; Tseng et al., 2001; Luttrell and Lefkowitz, 2002) (Figure 1.6). Several lines of studies have corroborated the crucial role of βARK and β-arrestin in receptor desensitization. For instance, it has been shown that a mutant form of β_2AR, which is a poor substrate for βARK, fails to be sequestered and desensitized upon stimulation (Ferguson et al., 1995). Conversely, the sequestration of this receptor can be restored by overexpression of βARK or β-arrestin (Tsuga et al., 1994; Ferguson et al., 1996). Homologous desensitization is thought to be faster acting compared to heterologous desensitization (Lefkowitz, 1993). Following receptor phosphorylation, the dysfunctional cell surface receptors enter the cells via a mechanism called receptor internalization (Tseng et al., 2001). This process involves the entering of the receptors to clathrin-coated vesicles through mediation of β-arrestins (Dzimiri, 1999)(Figure 1.6). Receptor internalization is crucial in the process of dephosphorylation and resensitization of the receptors (Sibley et al., 1986). Receptors in the vesicles contain less phosphate compared to those of the plasma membrane, reflecting high phosphatase activity in vesicles (Sibley et al., 1986). Subsequently, the dephosphated receptors may be recycled to the cell surface as active receptors, or may be directed toward lysosomes for degradation (Figure 1.7) (Hausdorff et al., 1990; Pierce and Lefkowitz, 2001; Luttrell and Lefkowitz, 2002). Lysosomal degradation of the receptors following sequestration might be viewed as a correlation between desensitization and further downregulation (Lefkowitz, 1998).

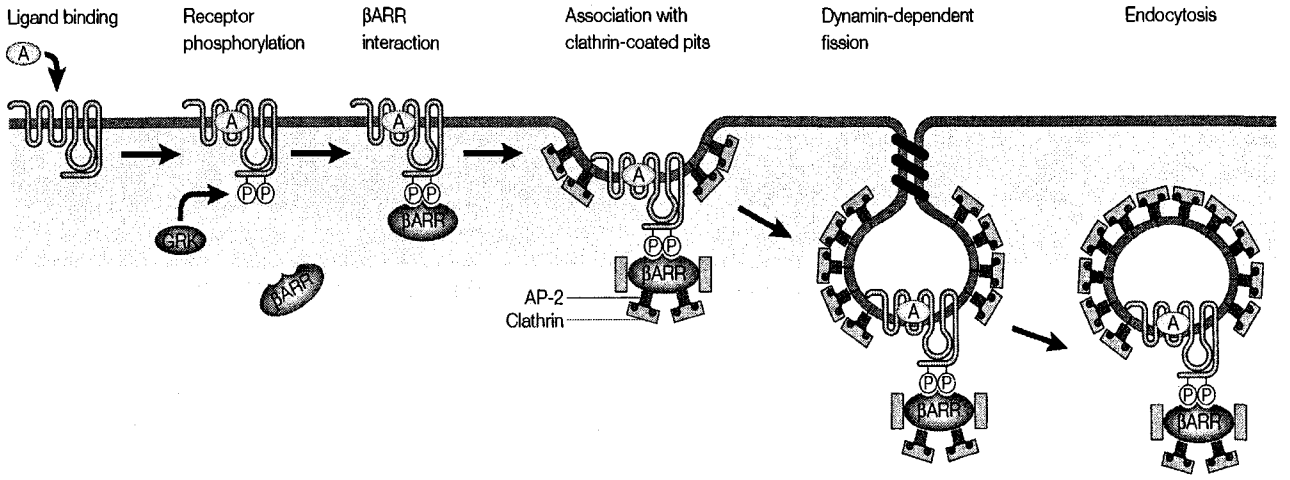


Figure 1.6: Schematic representation of receptor desensitization and internalization following agonist stimulation. Following receptor phosphorylation by G protein coupled receptor kinase (GRK), β-arrestin (βARR) binds to the phosphorylated receptor, further recruits clathrin, and targets the receptors for clathrin-mediated endocytosis. Adapted from Pierce et al (2001) Nature Reviews, 2:727-33.

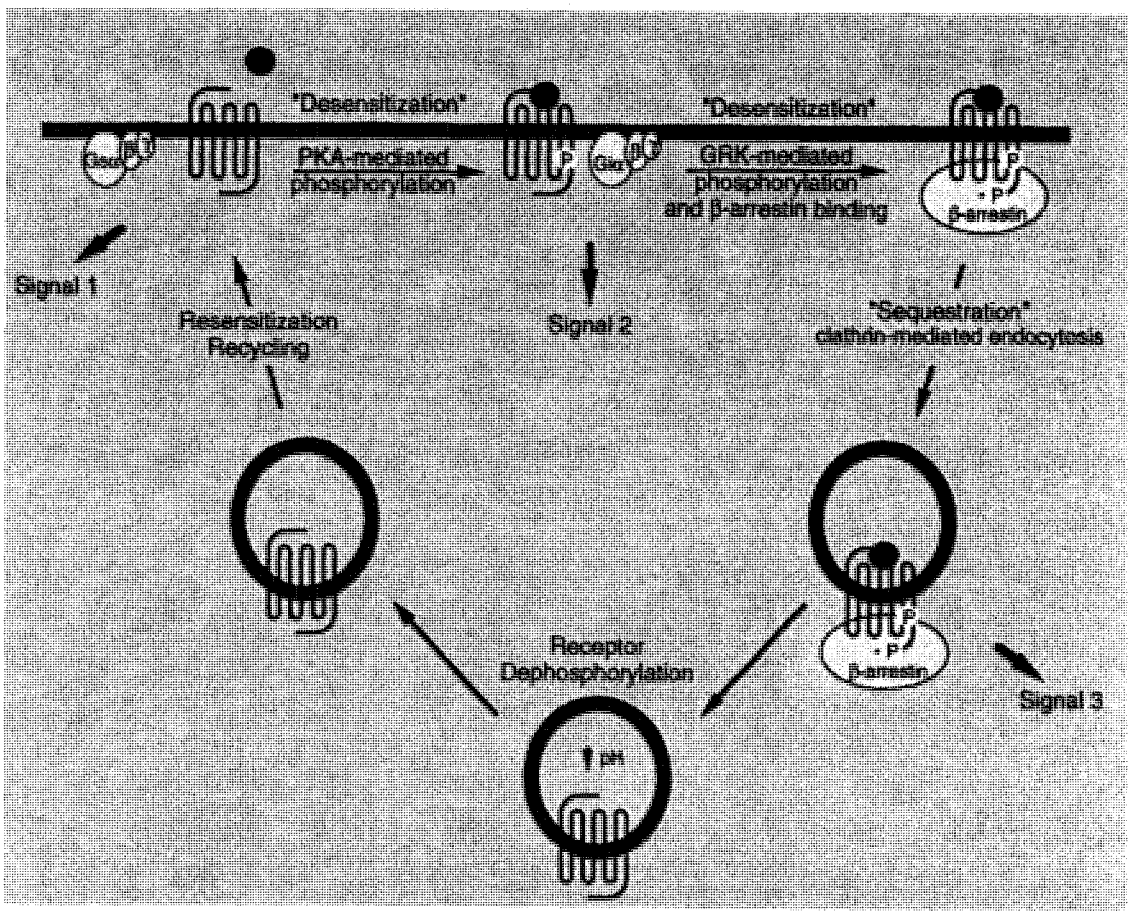


Figure 1.7: General illustration of receptor desensitization, internalization, dephosphorylation, and recycling. Following seconds to minutes of stimulation, PKA (heterologous desensitization) or GRK (homologous desensitization) phosphorylate the receptors. β-arrestin further binds to the phosphorylated receptors, stops the signalling, and further mediates internalization. Finally, receptors in vesicles are either transferred to lysosome for degradation or are recycled back to the cell-surface as functional receptors. Adapted from Lefkowitz (1998), Journal of Biological Chemistry, 30: 18677-80.

At normal state, a proper balance exists between receptor production and degradation (Tseng et al., 2001). However, as described below, an altered physiological environment including persistent stimulation of βAR can shift the balance toward more degradation and less protein production (Lefkowitz, 1998).

The process of rapid desensitization is also rapidly reversible. Within 15-30 minutes following removal of the agonist, the activity of the receptor and AC responsiveness was shown to reach normal state (Frederich et al., 1983; Hadcock and Malbon, 1991; Lefkowitz, 1998). However, if receptor stimulation continues for several hours to days, the receptors undergo a more severe regulation that eventually leads to partial loss of the cell surface receptors.

1.2.5.2 Receptor Downregulation

If the stimulation of the receptor persists for a long period of time (longer than four hours), two major compensatory responses occur: 1) reduction of the steady-state mRNA levels encoding βARs, or 2) enhancement of receptor degradation. It has been shown that chronic incubation of hamster vas deferens cells with isoproterenol results in decreased βAR mRNA levels via increased destabilization of the mRNA transcripts, such that the half-life of mRNAs declines from 12 hours in untreated cells to 5 hours in agonist-treated cells (Hadcock et al., 1989). The stability of mRNA (or the rate of transcription) is thought to be modulated by the level of the activation of the receptor, intracellular cAMP production, and functional PKA activity (Hadcock and Malbon, 1991).

Enhanced degradation of the internalized receptors also plays a crucial role in downregulation of the receptor. As discussed in section 1.2.5.1, the internalized receptor can be recycled to the cell surface after agonist-exposure or can move from endosomes to

lysosomes for degradation. The relationship between receptor internalization and downregulation is complex and is an overlapping point between desensitization and downregulation (Moore et al., 1999). It has been observed that in the downregulation process the recycling rate constant of internalized receptors is increased, which in turn promotes the duration of diversion of the receptors from endosomes into lysosomes for degradation (Moore et al., 1999 {Moore, 1999 #8110). Unlike the desensitization process, which is a rapid reversible process, downregulation is only reversed when new receptors are synthesized (Su et al., 1980). Agonist-affected cells require days to weeks to recover their full complement of newly expressed receptors (Hadcock and Malbon, 1991). In some cases the receptor density of the affected cells are recovered only by 50-90% (Wang et al., 1990). First reported by Bristow et al (Bristow et al., 1982), βAR downregulation is very well documented in heart failure, occuring as a result of SNS overactivity and persistent stimulation of βARs by NA (Bristow et al., 1982; Davis et al., 1988; Cohn, 1990).

In conclusion, phosphorylation of the receptor during short-term agonist stimulation or downregulation as a result of long-term exposure exert a negative feedback effect, blunting the cell response to further stimulation, and protecting the organ against the continuous attack of exogenous or endogenous agonists.

1.2.5.3 Differential Regulation of the βAR Subtypes in CHF

Several investigations have shown that the βAR subtypes do not necessarily undergo the same regulatory pathways under the same physiological or pathophysiological conditions. Corroborating this statement is the fact that in CHF β_1AR is selectively downregulated, while β_2AR is only uncoupled from G protein and its density remains unchanged

(Bristow et al., 1986; Brodde, 1991; Feldman, 1993; Moniotte et al., 2001). Furthermore, upon continuous stimulation, β_2AR is not only phosphorylated and uncoupled from G_s protein but is coupled to G_i protein as well (Daaka et al., 1997). G_i activation not only disrupts the concurrent G_s-mediated cAMP production, but is also postulated to exert antiapoptotic effects on cardiomyocytes through stimulation of different intracellular pathways (Zheng et al., 2004; Brodde et al., 2006). Therefore, switching the coupling of β_2AR from G_s to G_i possesses beneficial effects in promoting the survival of cardiomyocytes and protecting the heart against chronic stimulation of the receptors.

1.3 Diabetes Mellitus

1.3.1 General

According to the National Diabetes Surveillance System, 4.8% of Canadians aged 20 years and older have diabetes. Furthermore, it has been reported that diabetes is the seventh major cause of death in Canada. There are two types of diabetes; Type I and type II. Type I results from autoimmune destruction of pancreatic β-cells leading to deficient insulin production (Cardell, 2006; Daneman, 2006; Tsai et al., 2006). It accounts for 10% of all diabetes, and genetic makeup is thought to be the most prominent risk factor of the disease. However, the more common type of diabetes that accounts for ~90 of all diabetes is type II (Moller, 2001). The prevalence of type II DM is estimated to reach 200-300 millions worldwide by the end of the decade (Amos et al., 1997; Kopelman and Hitman, 1998). Type II DM is a complicated heterogeneous disorder with multiple risk factors and associated diseases. As discussed below, the insulin secretion is approximately normal, however, the peripheral organs are not responsive to insulin

(insulin resistance). Family history and life style are important risk factors, with obesity being the most prominent contributing factor leading to type II DM (Gress et al., 2000; Moller, 2001; Egan, 2003). The complications of diabetes are strongly related to high blood glucose levels and are mostly correlated with the duration of diabetes. These complications may include microvascular changes, resulting in retinopathy, nephropathy and neuropathy, or macrovascular changes leading to several types of cardiovascular diseases. It has been reported that cardiovascular diseases accounts for the majority of diabetic morbidity and mortality.

1.3.2 Insulin Signalling

Skeletal muscle, liver and adipose tissues are the major sites of insulin action, however insulin performs diverse roles in each of these organs. Insulin mediates glucose uptake and utilization in skeletal muscle. It suppresses glycogenolysis and lipolysis in the liver and adipose tissue respectively (Nonogaki, 2000; Roden, 2006). Initially, binding of insulin to insulin receptor (IR) activates the tyrosine kinase activity of the receptor, leading to autophosphorylation, as well as tyrosine phosphorylation of several IR substrates. These, in turn, interact with phosphoinositol 3-kinase (PI-3K), which is the major mediator of insulin signalling. It has been reported that inhibition of this kinase leads to severe derangement of insulin action in peripheral organs (Okada et al., 1994). PI-3K, by phosphorylating a number of protein kinases, mediates glucose uptake in the skeletal muscle. Protein kinases including protein kinase B (PKB) and two isoforms of protein kinase C (PKC), ζ and λ, act downstream of PI-3K to mediate glucose transport via enhanced glucose transporter 4 (GLUT 4) translocation from the endoplasmic

reticulum to the plasma membrane (Morisco et al., 2005). Regardless of the degree of importance of each of these kinases, it seems that the combination of PKC ζ/λ and PKB serves as a powerful mechanism in the regulation of glucose transport (Farese, 1996). It is hypothesized that the activity of key gluconeogenic enzymes in the liver such as phosphoenolpyrovate carboxykinase and glucose-6-phosphatase are inhibited by insulin, predominantly via suppression of gene expression, leading to suppressed glucose production (Barthel and Schmoll, 2003). Lipolytic pathways in adipocytes are also antagonized by insulin through PI-3K pathway (Okada et al., 1994). In health, insulin seems to use multiple but well-coordinated pathways to regulate glucose metabolism. Absence of such coordination leads to several metabolic abnormalities and onset of the diseases such as diabetes (Farese, 1996).

1.3.3 Pathophysiology of Type II Diabetes

Type II DM is a metabolic disorder mainly defined by abnormalities in carbohydrate and fat metabolism (Scheen, 2003). Multiple factors, various organs, and sequences of events are involved in the pathophysiology of type II DM. Type II DM is a bipolar disease state characterized by both decreased insulin sensitivity in peripheral tissues and depressed insulin secretion.

1.3.3.1 Obesity

Obesity, genetic makeup, age, and sedentary life style are considered important risk factors toward development of insulin resistance (Gerich, 1998; Scheen, 2000; Kahn, 2003). As several studies suggest, the major risk factor responsible for IR desensitization to insulin is increased FFA levels (Reed et al., 2000; Zhang et al., 2003; Srinivasan et al.,

2005; Li et al., 2006). In fact, obesity, especially intraabdominal adiposity, which is observed in the majority of type II DM subjects, is associated with changes in ambient FFA concentrations (Scheen, 2000). This condition suggests that a prominent association between obesity and type II DM is insulin resistance, which is potentially driven by enhanced fat availability. The mechanisms through which high levels of fat cause insulin resistance and impair glucose uptake and metabolism have been the subject of intensive investigations. Randle et al first described the concept of glucose-fatty acid cycle (Randle et al., 1963). According to this hypothesis, increased fatty acid oxidation due to increased substrate availability along with increased expression of FFA oxidation enzymes results in enhanced lipid-derived acetyl-coA, severely inhibiting the pyruvate dehydrogenase complex. Inhibition of this enzyme eventually leads to feedback reduction in glucose uptake. While the effects of lipid may in part be mediated by substrate competition through the glucose-fatty acid cycle, direct interference of lipid derivatives with insulin signal transduction pathways is also likely to play an important role (Schmitz-Peiffer et al., 1999; Itani et al., 2000; Schmitz-Peiffer, 2000). Multiple interferences of lipids with insulin action ultimately blunt the functions of insulin to retain glucose in skeletal muscle or to suppress hepatic glucose release (Iwanishi and Kobayashi, 1993; Rosholt et al., 1994; Belfiore and Iannello, 1998). Insulin resistance ultimately results in severe derangement of glucose regulation and onset of diabetes. The diabetic state is exacerbated by accumulation of lipids within pancreatic islets, which is proposed to impair insulin secretion and lead to progressive insulin deficiency (lipotoxicity) (Unger, 1995)

1.3.3.2 Insulin Resistance

Decreased insulin sensitivity or insulin resistance has been considered by far the cornerstone in glucose dysregulation and type II DM diagnosis (Scheen and Lefebvre, 1992; Goldstein, 2002). As mentioned above, insulin resistance is defined as the failure of target tissues to increase whole body glucose disposal in response to insulin. As such, while insulin production and secretion is normal, peripheral tissues such as skeletal muscle are unable to retain glucose. In this condition, deterioration of glucose tolerance can be prevented by increased capacity of pancreatic β-cells to secrete insulin. Thereby, normal glucose homeostasis can be maintained in a state of chronic hyperinsulinemia (Reaven, 1997). However, when insulin overproduction can no longer be fulfilled by β-cells, decompensation of glucose homeostasis occurs. As such, an early indication of type II DM is the presence of hyperinsulinemia, which is followed in later stages by hyperglycaemia due to partial failure of β-cells to secrete insulin.

1.3.3.3 Impaired Glucose Tolerance

Impaired glucose tolerance (IGT) is defined as glucose levels higher than normal but below the levels of diabetes (Long et al., 1994). In essence, insulin resistance is the predisposing factor to IGT. In the presence of insulin resistance, normal glucose tolerance can be maintained only if the β-cells modify the rates of insulin secretion. However, similar to type II DM, IGT is associated with some degrees of loss of pancreatic β-cell function, leading to inability to balance glucose levels. IGT is an intermediate state in the transition from normal glucose tolerance to type II DM (Abdul-Ghani et al., 2006). Variations in β-cell response seem to account for the differences in the severity of glucose intolerance observed in patients with IGT and type II DM

(Reaven, 1997). Subjects, although still normoglycaemic, exhibit dual complications of type II DM, ie. insulin resistance and impaired β-cell function. The risk factors for impaired glucose tolerance and type II DM overlap considerably and include insulin resistance, obesity, sedentary life style, and family history (Dagogo-Jack and Santiago, 1997). Impaired glucose tolerance may ultimately progress to type II DM and is associated with a high risk of CVD (Dagogo-Jack and Santiago, 1997).

1.3.3.4 Hyperglycaemia

As discussed in section 1.3.3.2, regardless of the factors involved in insulin resistance, the first response of the body is the elevation of insulin secretion from pancreatic β-cells. This compensatory hyperinsulinemia can maintain blood glucose at normal levels, the condition observed in prediabetic state (Mitrakou et al., 1992; Lillioja et al., 1993; Sawant et al., 2004). Overtime, when a number of pancreatic β-cells are no longer capable of secreting insulin to compensate for insulin resistance, apoptosis of a group of β-cells occurs, leading to decreased insulin secretion, thereby high levels of glucose are no longer modulated and diabetes occurs (Reed et al., 2000; Kostis and Sanders, 2005). It is suggested that through transition from insulin resistance to hyperglycaemia, 20-40% of the β-cells are lost, and insulin secretion is decreased (Ferrannini, 1998). Hyperglycaemia also directly interferes with insulin secretion by inducing islet autoimmunogens or by downregulating insulin gene expression, leading to further deterioration in insulin secretion (Moran et al., 1997; Wilkin, 2001).

1.3.3.5 Dyslipidemia

Altered lipid profile in the blood is another characteristic of type II DM. Dyslipidemia is defined as high triglyceride levels, low high-density lipoprotein concentration, and

elevated low-density lipoprotein. Chronic elevation of FFA contributes to altered lipid profile (Scheen, 2003). Dyslipidemia is significantly associated with coronary artery disease (Rau et al., 2007). Regulation of FFA metabolism aids to restore normal lipid profile.

1.3.3.6 Gene Variations

Recently, it has been found that gene polymorphism of β_3AR, a key receptor in the regulation of lipolysis and thermogenesis, is associated with altered receptor function and increased risk of obesity and insulin resistance (Strosberg, 1997; Hoffstedt et al., 2002). Certain variants of the β_2AR gene can also be involved in increased FFA, increased fasting insulin levels, and thereby enhanced susceptibility to type II DM (Carlsson et al., 2001). Figure 1.8 represents multiple factors involved in the pathogenesis of type II DM.

1.4 Animals Models of Diabetes: Zucker and Streptozotocin-Treated Rats

Several methods have been proposed to induce type II DM in animals. The genetic models of obesity and diabetes such as obese Zucker fatty rats are commonly used for studies of type II DM. They exhibit insulin resistance and hyperinsulinemia with normal glucose metabolism at early weeks of age, however glucose metabolism deteriorates over time, predisposing these animals to hyperglycaemia (Huisamen et al., 2001). This animal model has the disadvantage of prohibitive costs.

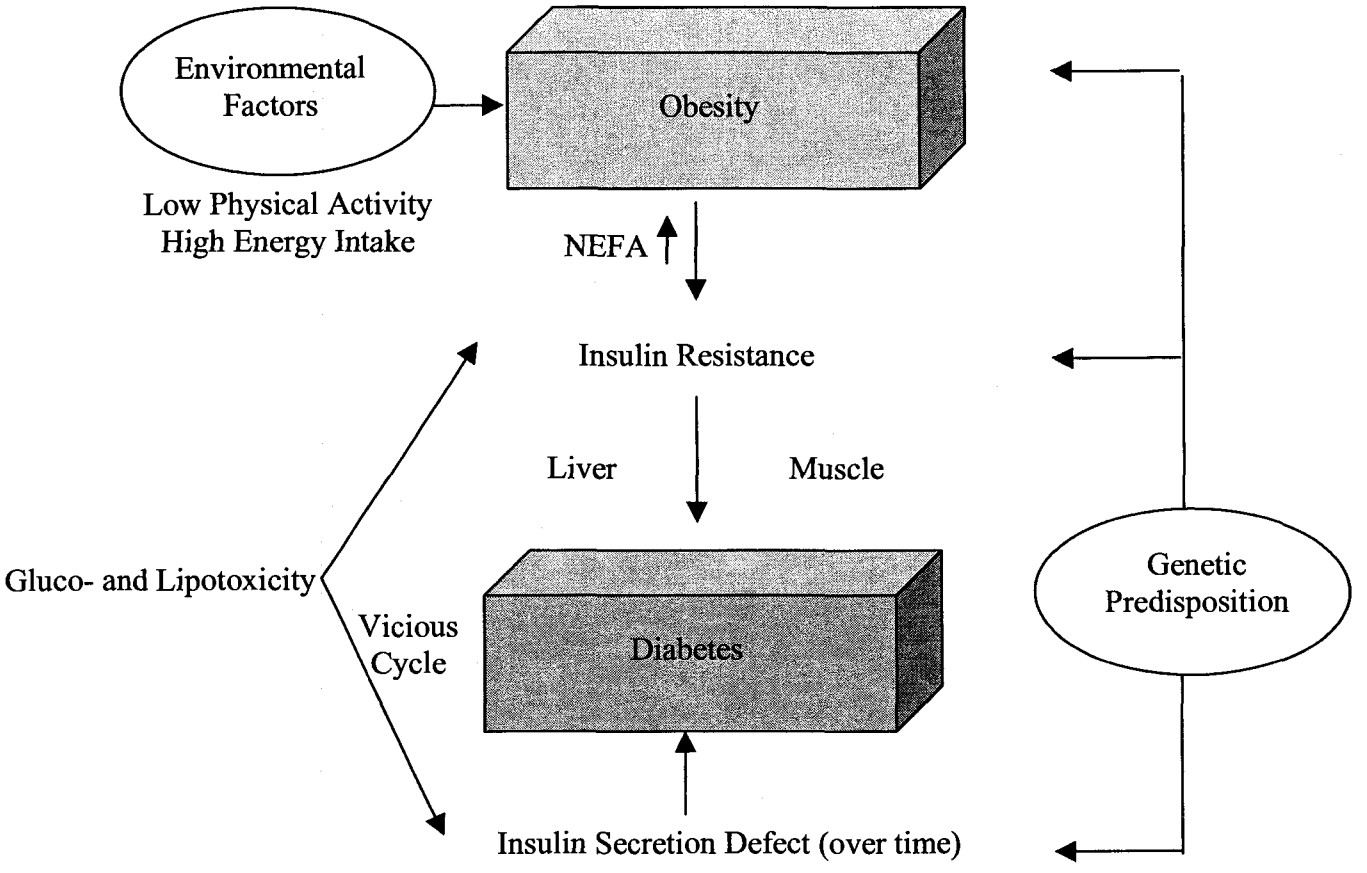


Figure 1.8: Schematic representation of the various factors involved in pathogenesis of type II DM. Insulin resistance is the core defect linking obesity to subsequent diabetes; environmental factors and genetic predisposition are contributed to both obesity and type II DM; decreased insulin sensitivity leads to decreased insulin secretion over time, which relates to progression of the disease. Modified from Scheen et al (2003), Acta Clinica Belgica, 58(6) 335-41.

As described in section 1.3.3.2, the hallmark of type II DM is insulin resistance. Conceivably, inducing insulin resistance can potentially lead to diabetes. A group of studies have generated insulin resistance in experimental models merely by providing high fat diet (HFD) (Kraegen et al., 1986; Storlien et al., 1986; Kraegen et al., 1991). This finding corroborates further the strong association of increased FFA levels with the onset of insulin resistance and diabetes. However, HFD models take several months to develop diabetes and yield only moderate hyperglycaemia.

One of the most common methods to induce DM is the injection of streptozotocin (STZ). STZ is an alkylating agent that is selectively taken up by pancreatic β-cells via GLUT-2 (Szkudelski, 2001). It induces its toxic effects through alkylating β-cell DNA and resulting cell death (Delaney et al., 1995; Elsner et al., 2000). STZ also acts as a nitric oxide donor, increasing islet destruction via enhanced free oxygen radicals (Morgan et al., 1994; Kroncke et al., 1995). Type I DM is commonly induced by intravenous injection of high dose of STZ (>60 mg/kg) (Ganda et al., 1976; Katsumata et al., 1992). It is thought that high doses of STZ destroy a vast majority of the cells leading to total loss of insulin production (Szkudelski, 2001).

For induction of type II DM using STZ, one method is to inject neonatal rats with moderate dose STZ that results in mild hyperglycaemia and glucose intolerance over approximately 8 weeks (Portha et al., 1979). Since this method is time consuming, an alternative approach is applied in older rats and with shorter time frame. Another method that was widely used in recent years by multiple groups to induce type II DM in a short period of time is the combination of HFD with a moderate dose STZ (45 mg/kg) (Reed et al., 2000; Sawant et al., 2004; Srinivasan et al., 2005). Consumption of HFD initiates a

state of insulin resistance. The administration of a moderate dose STZ (45 mg/kg) causes a partial destruction of pancreatic β-cells and a reduction of insulin secretion capacity, such that the subjects are no longer able to maintain normal blood glucose levels. As a result, severe hyperglycaemia occurs in these animals shortly following injection of STZ (Sawant et al., 2004). Contrary to the animal models of high dose STZ (Type I DM), utilizing a moderate dose of STZ (45 mg/kg) does not lead to total destruction of β-cells. Therefore, insulin production is not totally lost in this animal model. It has been demonstrated that glucose reaches normal values at fasting state, representing the presence of functional insulin when unopposed by glucose loading. This latter characteristic is the hallmark distinguishing this animal model from those of type I DM (Srinivasan et al., 2005). However, contrary to genetic models of diabetes, these animals are not obese. Although similar to genetic models, a clear reduction in insulin sensitivity is observed in both HFD STZ-treated rats and their HFD-fed vehicle controls. In fact, the sustained hyperglycaemia accompanied by insulin resistance in this animal model mimics the late stages of the clinical manifestation of type II DM. This model was applied in this project as an animal model of hyperglycaemia.

1.5 Altered Sympathetic Nervous System in Diabetes

It appears that SNS activity is altered in type II diabetic patients. Various autonomic nervous system dysfunctions such as depressed heart rate variability have been significantly correlated with the diabetic state (Bellavere et al., 1996; Tack et al., 1996; Paolisso et al., 2000). It is believed that several metabolic defects of diabetes particularly insulin resistance leads to SNS dysfunction (Tack et al., 1996; Paolisso et al., 2000;

Manzella et al., 2001; Manzella and Paolisso, 2005). Individuals with altered SNS function, on the other hand, exhibit impaired insulin sensitivity and thereby are more inclined to developing diabetes over the years (Paolisso et al., 1991; Carnethon et al., 2003; Kostis and Sanders, 2005). It is also possible that both insulin resistance and autonomic dysfunction have a shared origin such as obesity and physical inactivity (Ivy, 1997; Ito et al., 2001; Melanson and Freedson, 2001).

1.5.1 Link Between Noradrenaline and Metabolic Parameters

1.5.1.1Glucose

Glucose is a major source of energy of the cell and is produced mainly by the liver. Glucose has various interactions with the SNS. The SNS modulates hepatic glucose production by activating glycogenolysis in the fed state and accelerating gluconeogenesis in fasted states (Nonogaki, 2000). Moreover, glucose-stimulated insulin release from the pancreas is modulated by NA (Nonogaki, 2000). As discussed later, glucose translocation into cardiac and skeletal muscle requires short-term SNS activity and βAR stimulation (Czech and Corvera, 1999; Morisco et al., 2005). On the other hand, several lines of evidence indicate that glucose itself can affect SNS function under physiological or pathophysiological conditions. It has been demonstrated that glucose ingestion activates the SNS signalling (Landsberg and Young, 1978). Furthermore, a direct arterial infusion of glucose was shown to induce a significant increase in NA concentration, particularly in rats susceptible to obesity by HFD feeding (Levin and Sullivan, 1987; Levin et al., 1989). A similar finding has been reported in healthy humans wherein acute hyperglycaemia elevates muscle sympathetic nerve activity (Hoffman et al., 1999).

Glucose-induced NA release in hypoinsulinemic STZ-treated rats further implies that enhanced SNS activity following a glucose load is not due to secondary reflex hyperinsulinemia, but due to direct effect of glucose on the SNS (Levin and Sullivan, 1987). It is believed that the brain possesses glucose-sensitive neurons located in the hypothalamic and brainstem areas, that are capable of regulating the SNS (Stoddard-Apter et al., 1983). These hypothalamic neurons respond to hyper- or hypoglycaemic conditions by changes in their firing rates (Oomura, 1983). Hepatic glucoreceptors were also reported to activate SNS in response to hyperglycaemia via vagal afferent signals to the brain (Niijima, 1984). Accordingly, in type II DM, hyperglycaemia can alter the SNS activity. It has been shown that high fasting plasma glucose levels in pre-diabetic and diabetic patients are significantly correlated with depressed heart rate variability, implicating the presence of heightened SNS and/or reduced pSNS activity (Singh et al., 2000). Moreover, cardiac and plasma NA content has been reported to increase significantly in diabetic patients (Ferraro et al., 1993). As a subset of studies suggests, the metabolic control of diabetic rats with administration of insulin normalizes glucose levels, which is further associated with reduced NA levels and restored βAR density. These results confirm the important role of plasma glucose levels in the regulation of NA release in diabetes (Ramanadham et al., 1983; Ganguly et al., 1987).

1.5.1.2 Insulin

As a consequence of insulin resistance, hyperinsulinemia has been related to disorders such as obesity, type II DM and hypertension (Tack et al., 1996). Several clinical trials denoted insulin as a highly effective stimulator of the SNS. It has been reported that in normal subjects infusion of insulin in a dose-dependent manner increases the systolic

blood pressure, heart rate and NA levels, indicative of enhanced SNS activity (Anderson et al., 1991; Kern et al., 2005). Insulin acts via direct stimulation of the central nervous system to activate the SNS (Fisher et al., 2005; Manzella and Paolisso, 2005). In fact, chronic stimulation of the SNS due to chronic hyperinsulinemia is an important factor in explaining the increased incidence of CVD in type II DM (Tack et al., 1996). In addition, activation of the SNS can impair insulin sensitivity. Walters and colleges demonstrated that in normal subjects, infusion of NA in physiological range impairs acute disposal of an intravenous glucose load, significantly decreasing glucose tolerance and insulin sensitivity (Walters et al., 1997). In an indirect support of this clinical finding, an experimental study demonstrated that βAR-less mice which lack the ability to respond to NA exhibit higher insulin sensitivity and glucose disappearance rate after a glucose challenge (Asensio et al., 2005). The cross-talk between insulin signalling and the SNS is complex. The SNS appears to have direct role in glucose transport into cardiomyocytes via enhanced PKB phosphorylation (Huisamen et al., 2001; Morisco et al., 2005). As such, it appears that insulin and SNS perform their role through overlapping pathways. However, if stimulation of βAR persists, PKB phosphorylates IR, inhibits tyrosine autophosphorylation, further reduces insulin mediated glucose transport, and consequently initiates a state of insulin resistance (Morisco et al., 2005).

Moreover, SNS-mediated vascular tone of perfusing arterioles plays an important role in the delivery of glucose and insulin to peripheral organs (Jacob et al., 1996). Hence, continuous SNS stimulation of α-adrenergic vascular receptors leads to arteriolar vasoconstriction, blunting the blood flow away from skeletal muscle, and eventually decreases insulin efficiency and glucose disposal (Palatini and Julius, 1997; Kostis and

Sanders, 2005). This is further evident in hypertensive patients who are usually diagnosed with impaired insulin-stimulated glucose disposal and resulting hyperinsulinemia (Jacob et al., 1996).

1.5.1.3 Free Fatty Acid

Another important metabolic alteration in diabetes is the elevation of FFA. Increased FFA is linked to sympathetic activation and subsequent cardiovascular alterations. Paolisso et al have shown that elevated plasma FFA in normal rats exaggerates cardiac SNS activity as indicated by depressed heart rate variability and increased plasma levels of NA (Paolisso et al., 2000). Similarly, altered cardiac function in diabetic patients contributes to enhanced FFA levels (Manzella et al., 2001). The neural pressor response is the most likely mechanism to explain the FFA-mediated SNS activation (Paolisso et al., 2000; Gadegbeku et al., 2002). The interrelation of FFA and SNS signalling functions in reverse, such that SNS can stimulate the release of FFA. A study on healthy subjects demonstrated that infusion of NA causes a 40% increase in plasma FFA via enhanced lipolysis in adipocytes (Marangou et al., 1988). FFA, on the other hand, exacerbates insulin resistance by inhibition of peripheral glucose uptake (Paolisso et al., 1994; Randle et al., 1994; Boden, 1999). This complex relation between FFA, SNS activity, and insulin sensitivity highlights the possible role of high fat intake, obesity and physical inactivity as shared precursors of insulin resistance and autonomic dysfunction (Carnethon et al., 2003).

1.5.2 Diabetic Autonomic Neuropathy

One of the most significant impacts of high glucose levels on autonomic dysfunction in diabetes is the development of diabetic autonomic neuropathy (DAN). DAN, defined as specific damage of autonomic nerve fibers, is a common complication of diabetes and can extensively alter SNS function (Debono and Cachia, 2007). Hyperglycaemia is specifically damaging to neurons by inducing the formation of reactive oxygen species and reducing the capacity of neurons to buffer free radicals (Sullivan and Feldman, 2005). Hyperglycaemia exacerbates DAN further by reducing the production of growth factors which are important for the optimal function of the nervous system (Schmid et al., 1999). In general, DAN is associated with decreased sympathetic innervation (Stevens et al., 1998). Cardiac sympathetic dysinnervation has been repeatedly reported in diabetes, having been significantly correlated with cardiovascular complications such as sudden cardiac death (Turpeinen et al., 1996; Stevens et al., 1998; Stevens et al., 1998; Schnell et al., 2002). Radiolabelled analogues of NA, which are retained by sympathetic nerve terminals, are relatively novel tools that allow the evaluation of sympathetic nerve integrity in DAN (Stevens et al., 1998). For instance, the NA analogue [^{11}C] *meta*-hydroxyephedrine ([^{11}C]HED) retention was shown to be decreased in diabetic heart, implicating increased NA concentration in presynaptic areas. This [^{11}C]HED decrease in DAN was suggested to be specifically related to impaired neuronal tracer uptake due to NAT-1 dysfunction or neuronal loss (Stevens et al., 1998). Appropriate glycaemic control over a four-year follow up has been demonstrated to improve sympathetic innervation in type I DM, as observed by increased global [^{123}I] *meta*-iodobenzylguanidine ([^{123}I]MIBG) uptake (NA analog), although the indirect cardiac

autonomic function tests did not show any improvement over this period (Ziegler et al., 1998). As such, hyperglycaemia produces DAN, which in turn alters cardiac function through altering NA levels and modifying adrenergic receptor signalling (Debono and Cachia, 2007).

1.5.3 Altered β-Adrenergic Signalling in Diabetes

1.5.3.1 Myocardium

Although altered SNS activity has been proposed through various clinical and experimental studies, less information is available regarding the specific alterations occurring at the receptor and post βAR signalling levels. βAR signalling in type I DM has been widely investigated and since diabetes exhibits a strong incidence with occurrence of cardiovascular diseases, myocardial βARs have been the main focus of the published works (Ingebretsen et al., 1983; Williams et al., 1983; Nishio et al., 1988; Pagani et al., 1988; Roth et al., 1995; Matsuda et al., 1999). It has been observed that βAR density and βAR-mediated AC activation and cAMP production are reduced (Williams et al., 1983; Ramanadham and Tenner, 1987; Nishio et al., 1988; Beenen et al., 1997). Moreover, it is suggested that different subtypes of βAR undergo different alterations in their expression and regulation during the course of diabetes, further highlighting the specific mechanisms of actions underlying these alterations (Dincer et al., 1998; Dincer et al., 2001; Sellers and Chess-Williams, 2001).

1.5.3.2 Adipose Tissue

Altered βAR signalling in adipocytes and skeletal muscle has also received considerable attention in diabetes. Several studies have revealed that β_3AR agonists possess potent

anti-obesity and anti-diabetic effects in rodents (Arch and Wilson, 1996; Liu et al., 1998; Sasaki et al., 1998). Chronic treatment of obese rat and mice models with selective β_3AR agonists was shown to normalize hyperglycaemia, reduce hyperinsulinemia and circulating FFA, improve glucose tolerance, and cause a general improvement in glycaemic control (Arch and Wilson, 1996; Liu et al., 1998). The mechanisms underlying the anti-diabetic effects of β_3AR agonists are not fully elucidated. It is known that β_3ARs regulate FFA levels by utilizing fat droplets as major substrates of thermogenesis (Liu et al., 1998). The increased thermogenic capacity of brown adipocytes following β_3AR agonist treatment is further evidenced by increased expression of UCP1 and increased total tissue cytochrome oxidase activity (Liu et al., 1998; Sasaki et al., 1998). β_3AR agonists are considered as future candidates for treatment of obesity and diabetes in humans (An and Rodrigues, 2006).

1.6 Cardiovascular Diseases in Diabetes

Diabetes is associated with 2-3 fold increases in the risk of cardiovascular morbidity and mortality (Wilson et al., 2005). The diabetic state imposes various alterations to the cardiovascular system, linked directly or indirectly to insulin resistance and glucose abnormalities (Reaven, 1997). Dysregulation of metabolic control is associated with hyperinsulinemia, glucose intolerance and dyslipidemia, all related to poor symptomatic status in patients with CHF (Suskin et al., 2000). Moreover, insulin resistance is reported to be a major characteristic of CHF wherein heart failure patients exhibit decreased peripheral insulin sensitivity and increased fasting insulin levels (Swan et al., 1997; Kostis and Sanders, 2005).

Hyperglycaemia and impaired fasting plasma glucose levels have been significantly correlated with impaired autonomic control of heart function and depressed heart rate variability (Singh et al., 2000). Furthermore, as reported in an alloxan-induced glucose intolerant dog, high levels of glucose elicit myocardial chamber stiffness and reduce cardiac performance due to increased concentration of glycosylation end products bound to collagen (Avendano et al., 1999). Hyperglycaemia also induces damage to cardiomyocytes through generation of reactive nitrogen and oxygen (Fonarow and Srikanthan, 2006). However, the major impact of hyperglycaemia to CVD is probabely caused by endothelial dysfunction (Turner et al., 1998; Ajjan and Grant, 2006).

One of the prominent associations of DM and CVD is observed in DAN (Debono and Cachia, 2007). As discussed earlier, DAN disturbs the control of heart rate and contractility and vascular dynamics, and is invoked as a cause of sudden cardiac death in diabetes (Stevens et al., 1998; Stevens et al., 1998). In fact, the increased rate of diabetic cardiovascular mortality is five times higher in the presence of autonomic neuropathy, and sub-clinically, it can occur at very early stages of the disease (Debono and Cachia, 2007).

Another major factor that affects cardiac performance is alterations in metabolic activity. In normal heart, FFA oxidation accounts for 70% of ATP production, although the heart is capable of utilizing other substrates as well (Borradaile and Schaffer, 2005; An and Rodrigues, 2006). For instance, under stress or increased energy demand the heart relies mainly on glucose oxidation for ATP production (Taegtmeyer et al., 2002). In both type I and type II DM, glucose uptake, glycolysis, and pyruvate oxidation are impaired with concomitant two-fold increase in FFA oxidation, exhibiting the further shift to FFA

utilization (Borradaile and Schaffer, 2005; Fonarow and Srikanthan, 2006). Deficient glycolysis and increased FFA oxidation causes dysfunctional left ventricular dilation and onset of cardiomyopathy (Fonarow and Srikanthan, 2006). The high rate of FFA oxidation also increases oxygen demand along with generation of reactive oxygen species that can be potentially damaging to cardiac tissue (An and Rodrigues, 2006).

1.7 Therapeutic Interventions

At present, therapies for type II DM mainly focus on a combination of aggressive control of hyperglycaemia and improved insulin sensitivity. Anti-diabetic drugs, including sulphonylureas derivatives, biguanides, acarbose, and thiazolidinedions (TZDs), act on specific targets to enhance glycaemic control and improve symptoms. Sulphonylureas stimulate insulin secretion from pancreatic islets (Moller, 2001). Acarbose interferes with gastrointestinal glucose absorption, suppressing glucose production, and thereby reducing postprandial glucose levels (Moller, 2001). Biguanides such as metformin exert anti-hyperglycaemic effects mainly via decreased glucose output and enhanced insulin sensitivity and glucose turnover. It decreases glucose output through suppressing gluconeogenesis and glycogenolysis in the liver, and increases insulin sensitivity and glucose uptake via enhanced glucose tranporters in skeletal muscle (Wiernsperger and Bailey, 1999). In addition, it is believed that metformin improves insulin action through a general decrease in FFA oxidation, and thereby correcting any imbalance of the glucose-FFA cycle (Fischer et al., 1995). TZDs including pigliotazone and rosiglitazone are a class of insulin sensitizing drugs that provide an effective approach for treating type II DM. These drugs elicit their effects through stimulating peroxisome proliferators-

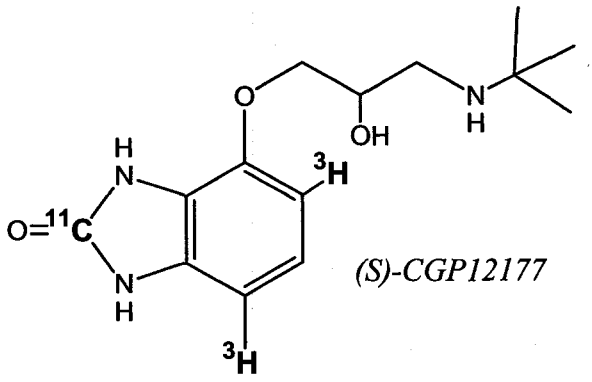
activated receptor-γ (PPAR-γ) and further mediating the expression of a variety of genes involved in insulin action and glucose and lipid metabolism (Kim et al., 2003; Yue et al., 2005). For instance, piglitazone improves insulin sensitivity via enhanced IR substrates and GLUT 4 expression. The anti-diabetic effects of this drug further include ameliorating lipid metabolism, reducing FFAs and inhibiting gluconeogenesis (Ding et al., 2005). In addition, metformin and TZDs have been demonstrated to improve cardiac function, partly owing to increased cardiac insulin sensitivity, decreased FFA levels and improved glucose oxidation (Khandoudi et al., 2002; Sidell et al., 2002; Kim et al., 2003; Yue et al., 2005). By modulating glucose metabolism, these drugs revert glucose, insulin, and FFA levels to normal values, thereby normalizing metabolic regulation of SNS activity and NA release, partly restoring normal physiology, and consequently preventing various complications of diabetes due to SNS alteration.

1.8 Radiotracer Used for βAR

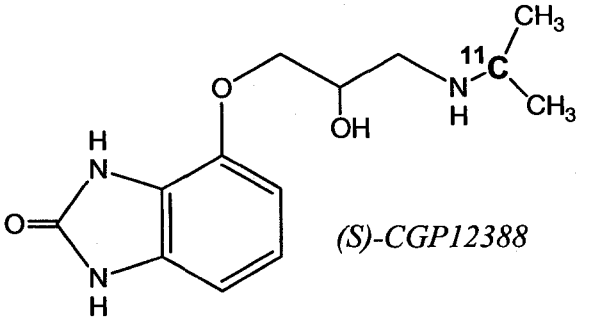
1.8.1General

Radiolabelled tracers are commonly used to assess the role of the receptors in normal physiology and diseases (Law, 1993). A number of radiolabelled βAR antagonists are considered proper candidates for pharmacological evaluation of the receptors. Antagonists including [^{125}I]-iodocyanopindolol ([^{125}I]ICYP), [^{125}I]-iodohydroxybenzylpindolol ([^{125}I]IHYP) and [^{3}H]dihydroalprenolol ([^{3}H]DHA) have been successfully used in βAR binding experiments to study various tissues (Savarese and Berkowitz, 1979; Ingebretsen et al., 1983; Kashiwagi et al., 1989; Tsuchihashi et al., 1989). (S)-CGP12177 (4-(3-butylamino-2-hydroxy-propoxy)-benzimidizol-2-one) is

another βAR antagonist that is widely used for the evaluation of receptor density. Unlike DHA, CGP12177 only binds to cell surface receptors due to its low lipophilicity (Log P=1.81) (Staehelin and Hertel, 1983), and therefore lacks the ability to measure internalized or vesicular receptors (Hertel et al., 1983). The dissociation rate constant (K_d) of the S- enantiomer for binding to βARs is at least 2 fold lower than that of R-enantiomer (Affolter et al., 1985). The tracer has high affinity for the βARs, and demonstrates similar selectivities for β_1- and β_2AR (K_d=0.3 nM β_1 and 0.9 nM β_2) (Nanoff et al., 1987). The uptake of the tracer can be specifically blocked in the presence of β-blockers, confirming the specificity of the binding to βARs. (S)-CGP12177 can be labelled with ^{3}H or ^{11}C (Figure 1.9). (S)-[^{3}H]CGP12177 is a widely used radiotracer for the evaluation of myocardial receptors in normal and disease states (Mauz and Pelzer, 1990; Ueki et al., 1993; Kitagawa et al., 1995). It also exhibits high uptake in the lung owing to the high expression of βAR in this tissue (Van Waarde et al., 1992; Law, 1993). Using (S)-[^{3}H]CGP12177, the receptor expression and function have been detected in brown adipocytes in obese subjects (Charon et al., 1995; Hoffstedt et al., 2002). (S)-[^{3}H]CGP12177 has also been employed to measure the receptor density in skeletal muscle (Sillence et al., 2005), or to analyse receptor regulatory pathways in kidney cells (Moore et al., 1999). Taken together, this tracer has been validated to assess receptor density in a variety of tissues, and therefore, its use was pursued in this project.



(S)-CGP12177



(S)-CGP12388

Figure 1.9: Molecular structure of (S)-CGP12177 and (S)-CGP12388. The [^{3}H] form of (S)-CGP12177 is used for the quantification of βARs in animal models. The [^{11}C] labelled form of (S)-CGP12177 and (S)-CGP12388 are used as the PET ligands for quantification of βARs in humans. Locations of ^{3}H and ^{11}C labels are indicated with the bold font style.

1.8.2 Positron Emission Tomography using (S)-[^{11}C]CGP1277 and (S)-[^{11}C]CGP12388

Positron Emission Tomography (PET) is a relatively novel imaging method for quantitative measuring of the receptors densities, energy metabolism or blood flow (Elsinga et al., 2001). Various adrenergic antagonists, including (S)-[^{11}C]CGP12177 and [^{11}C]CGP12388 (Figure 1.9), have been extensively used for characterization of βAR density in physiological and pathophysiological conditions. PET has the benefit to evaluate the time course of changes in the receptor density before, during and after pharmacological interventions, and can be used to guide therapy (Dubois et al., 1997). (S)-[^{11}C]CGP12177 is produced by reaction of [^{11}C] phosgene with the (S)-diamine precursor (Link et al., 2003). However, the production of [^{11}C]CGP12177 is very demanding, as the development of [^{11}C] phosgene requires highly controlled conditions (Elsinga et al., 2001). An alternative for [^{11}C]CGP12177 is the use of (S)-4-(3-(2`-[^{11}C] isopropylamino)-2-hydeoxy-propoxy)-2H-benzimidazol-2-one ((S)-[^{11}C]CGP12388), which is the isopropyl analog of (S)-CGP12177. This tracer can be more easily labelled from [^{11}C] acetone (Figure 1.9) (Elsinga et al., 1997; Doze et al., 2002). (S)-[^{11}C]CGP12388 exhibits similar characteristics to (S)-[^{11}C]CGP12177 and therefore, has been widely used in recent years for cardiac and pulmonary quantifications of the receptor density .

Using [^{11}C]CGP12177 in PET trials, it has been found that βAR density is approximately 12.7 pmol/g in normal human heart (Delforge et al., 2002). The first study measuring βAR density with PET in pathophysiological conditions showed a decreased βAR density in a group of patients with CHF due to idiopathic dilated cardiomyopathy (Merlet et al., 1993)(Figure 1.10).

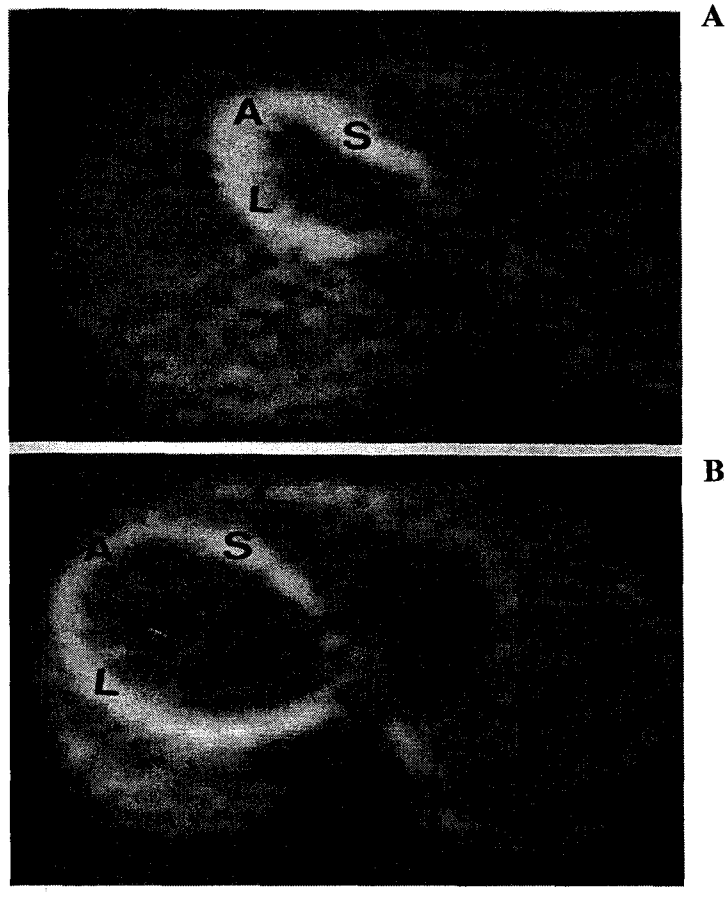


Figure 1.10: PET imaging of the heart using (S)-[^{11}C]CGP12177 in healthy subjects **(A)** and in patients with idiopathic dilated cardiomyopathy **(B)**. Adapted from Merlet et al 1993, Circulation, 97 (4): 1169-1178

Similarly, the B_{max} values measured with the same radiotracer in patients with hypertrophic cardiomyopathy have demonstrated βAR downregulation (B_{max}=7.70 pmol/g) (Lefroy et al., 1993).

In parallel, the adrenergic receptors have also been evaluated in the lung using PET. Pulmonary βAR density measured by PET after injection of [^{11}C]CGP12177 was 14.8 pmol/g in normal lung (Ueki et al., 1993). PET scanning of a group of patients with pulmonary tumors has demonstrated βAR downregulation in malignant areas (B_{max}=5.1pmol/g) (Qing et al., 1996). Both ^{11}C and ^{3}H labelled forms of the tracer are stable in vivo. It has been demonstrated that 93% of the (S)-[^{3}H]CGP12177 is excreted unchanged from the urine in anesthetised rats (van Waarde et al., 1992). A detailed study of (S)-[^{3}H/^{11}C]CGP12177 in dogs and rats failed to identify labelled metabolites with high performance chromatography in the plasma up to 80 minutes following injection (Jones et al., 1991). In summary, PET appears to be an accurate technique for measurements of the βAR density in cardiac and pulmonary diseases, and the results have been in agreement with in vitro data (de Jong et al., 2002).

1.9 Hypotheses and Objectives

1.9.1 General Goal

The *general* goal of this project is to examine the impact of hyperglycaemia on SNS signalling at the βAR site in a rat model of hyperglycaemia in various tissues, including the heart.

1.9.2 Hypotheses:

i) Sustained hyperglycaemia will impair SNS signalling.

ii) Repeated NA stimulation results in reduced binding to βARs.

iii) βAR antagonist (S)-[^{3}H]CGP12177 will measure specific binding to βARs in vivo in various tissues. Reduced myocardial βAR binding is expected in hyperglycaemic rats.

1.9.3 Primary Objective:

Delineate differences in βAR binding in vivo in normal and hyperglycaemic rats at two different time points using (S)-[^{3}H]CGP12177.

1.9.4 Specific Objectives:

i) Develop methods to measure in vivo (S)-[^{3}H]CGP12177 specific binding to βARs in various tissues.

ii) Perform subchronic treatments with βAR agonist isoproterenol to assess the capability of our procedure to measure reduced βAR binding following persistent βAR agonist stimulation.

iii) Develop the STZ-treated HFD animal model of sustained hyperglycaemia, and perform biodistribution studies using (S)-[^{3}H]CGP12177 at two time points (10 days and 56 days post-STZ).

iv) Establish the presence of specific binding of the tracer to βARs in STZ-treated animals.

1.10 Novelty of the Project

The cardiac SNS activity, βAR density, and post-receptor signaling have been widely investigated in the rat models of type I DM. However, the impact of diabetes on the receptor integrity in type II DM is underexplored. This project aims to investigate in vivo alterations in receptor binding in a rat model believed to exhibit human manifestation of type II DM. These experiments will help to elucidate whether there is a relationship between sustained hyperglycaemia and altered cardiac receptor binding in type II DM. More importantly, to our knowledge, we are the first to evaluate the in vivo binding profile of (S)-[^{3}H]CGP12177 in cardiac regions in diabetic rats. Previous in vivo biodistribution studies using this tracer has been performed primarily in normal rats to assess the validity of the tracer and methodology as a PET ligand. Hence, the results of this project lay the groundwork for future works in humans using (S)-[^{11}C]CGP12177 PET to evaluate the receptor integrity in diabetes.

2.0 MATERIALS AND METHODS

2.1 Animals, Drugs and Materials

2.1.1 Animals

Animal experiments were conducted in accordance with the recommendations of the Canadian Council on Animal Care and with approval from the Animal Care Committee at the University of Ottawa. Male Sprague-Dawley rats (Charles River Canada; Montreal, QC) were housed single or in pairs and maintained on a 12 h light/dark cycle with free access to food and water.

Normal male Sprague-Dawley rats (150-200 g) were used for characterization of the tracer. The rats were maintained on regular chow diet (Harlen Teklad 2019), consisting of 9% fat, 67% carbohydrate, 19% protein, and 5% fibre. Animals undergoing STZ treatment and their age-matched controls were initially fed regular chow diet upon arrival (5 weeks old, 150-175 g) from the breeder for five days. Following this period of environmental stress adjustments, the diet was changed to HFD (Research diets D12266B), with the following composition: 32% fat, 51% carbohydrate, 17% protein.

2.1.2 Drugs

(S)-[^{3}H](-)-CGP12177 (specific activity 43-59 Ci/mmol) was purchased from Amersham (U.K). (S)-CGP12177-HCl, propranolol-HCl, and isoproterenol-HCl, and STZ-HCl were obtained from Sigma. The drugs were dissolved in 0.9% sterile NaCl solution.

2.1.3 Materials

Osmotic minipumps were purchased from Alzet (US, Cupertino, CA). Solubilizer and scintillation cocktail (Hionic-fluor) were purchased from Amersham (UK). Isopropanol was purchased from Ricca chemical (US, Arlington, Tex), and H2O2 was purchased from Columbus chemical Industry (US, Columbus, OH).

2.2 Biodistribution

2.2.1 General Procedure

Biodistribution experiments were performed at approximately the same time of the day using an adaptation of the methods described by van Waarde (van Waarde et al., 1992). Briefly, restrained animals were injected with 8 μCi of (S)-[^{3}H]CGP12177 (49 Ci/mmol) diluted in 0.2 mL saline (pH=4.5-6.5) via a tail vein, dilated under a heat lamp. Animals were then sacrificed at 5, 15, 20, 30 or 45 minutes following tracer injection. Documented pharmacological studies favour a 30 minutes post-tracer time point (van Waarde et al., 1992; Law, 1993), and initial time course evaluation performed in our laboratory supported further this time period. Subsequent experiments were carried out using 30 minute sacrifice time. Myocardial tissue regions (100-150 mg) (right and left atria, right and left ventricle, and intraventricular septum), samples (100-150 mg) of the liver, kidney, skeletal muscle, brown adipose and white adipose tissue and pancreas were dissected out and placed in pre-weighted 20 mL polyethylene scintillation vials. Samples of trunk blood were collected in heparin- or ethylenediaminetetraacetic acid (EDTA)-coated tubes to prevent coagulation, and 100 μL samples were taken for radioactivity quantification. Blood samples were centrifuged using a benchtop centrifuge (Jouan,

CR3i) at 4000 rpm for 5 minutes to collect plasma. 100 μL of plasma were taken to determine radioactivity levels and the remainder stored at -80°C for insulin determination.

2.2.2 Optimization of Methods for Quantification of Tissue-Retained Radioactivity

In order to measure radioactivity levels using the scintillation counter, the tissues that have been dissected out in biodistribution procedures need to be dissolved and decolourized. The methodology used to process the tissues was adapted from previously published techniques (van Waarde et al., 1992). Briefly, 1 mL solubilizer was added to the 100 mg tissue samples and the mixture placed in an incubator-shaker (200 rpm) for 3-4 hours (temp 50-60°C). To decolourize samples, various volumes of hydrogen peroxide (H_2O_2, 30%,CCI) were tested (0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL). The same volumes of isopropanol were also added to inhibit the foaming caused by H_2O_2. While lower amounts of H_2O_2 and isoproranol (0.2 mL) were found not to be efficient for mixture decolourization, higher amounts (>0.6 mL) caused chemiluminescence (random single photon emissions produced by chemical interactions that register incorrectly as counts). As such, 0.4 mL was determined to be the optimal amount of both H_2O_2 and isopropanol for decolourization. For processing the blood, higher amounts of decolourizing agents were required (0.6 mL H_2O_2 / isopropanol), with no need to apply solubilizer. All samples were incubated for 30 min at 50-60°C to complete the reaction, resulting in a clear, slightly yellowish fluid. Glacial acetic acid (60 μl) was added to the vials to buffer the solution and reduce chemiluminescence, prior to addition of 10 mL of scintillation fluid (BCS-NA, Amersham). Figure 2.1 summarizes the steps used in the biodistribution.

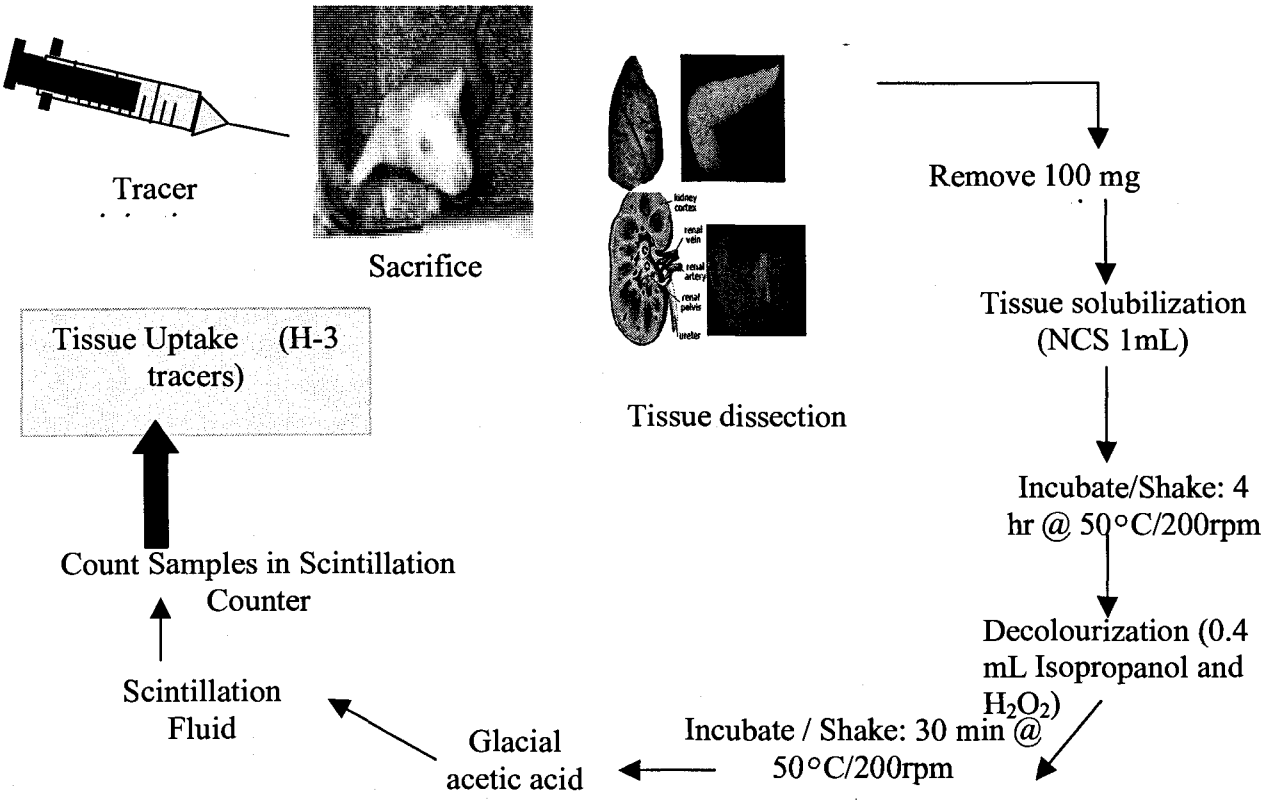


Figure 2.1: Schematic representation of the biodistribution procedure. At 30 minutes post tracer injection rats were sacrificed and tissues of interest were dissected out and placed in the vials. Tissues were then solubilized with 1 mL solubilizer followed by 4 hours incubation. Decolourizing agents were then added. The procedure was followed by a 30-minute incubation, and addition of glacial acetic acid and scintillation fluid. Finally, samples were counted using scintillation counter.

In summary, the *scintillation solution* developed to process the tissues contained: 1 mL solubilizer, 0.4 mL isopropanol, 0.4 mL H_2O_2, 60 µL glacial acetic acid, and 10 mL scintillation fluid. Finally, samples were vortexed for 1-2 minutes and counted using a Rackbeta scintillation counter, set to count the samples for 240 seconds, in duplicates. Radioactivity was expressed as counts per minutes (cpm).

2.3 In Vitro (S)-[^{3}H]CGP12177 Method Validation

2.3.1 (S)-[^{3}H]CGP12177 Standard Curve

To measure the radioactivity levels of the tracer and relate radioactivity dose to the counts registered in the scintillation counter (cpm), a standard curve was constructed. (S)-[^{3}H]CGP12177 (0.01 – 0.1 µCi) was added to the scintillation solution containing all the solubilizing and decolourizing agents listed above and activity counted as described.

2.3.2 Effect of External Light on Counting Efficiency

To determine the impact that ambient light may have on the scintillation, samples used in the creation of the standard curve were prepared in duplicate, and counted after 12 hours in the light or dark environment. The curves resulting from the samples were then compared.

2.4.3 Tissue Radioactivity Counts of (S)-[^{3}H]CGP12177: Effect of Colour

Following solubilisation and decolourizaton, most tissues appeared as clear liquids, however, a subset of the tissues was still slightly colourized. To address the effect that the colour of the tissue may have on tissue counts, solutions prepared using the range of

doses of radiotracer (0.01 – 0.1 μCi) were counted in the presence and absence of a highly colourized tissue (liver, 100 mg).

2.4.4 Tissue Radioactivity Counts of (S)-[^{3}H]CGP12177: Correction Factors

Due to the significant effect of tissue colour on the counts (section 2.4.3), correction factors were needed to obtain meaningful quantification of radiotracer distribution. To create these correction factors, six normal Sprague-Dawley rats were sacrificed and the tissues of interest were dissected out and processed as described in section 2.2.2. A solution containing 0.1% of the injected dose of radiotracer (0.008 μCi) was prepared. 80 μl of the radiotracer solution was added to each dissected tissue sample and processed as described above, with another 80 μl counted without any tissue present. An average of the six counts was taken for each tissue and compared to the standard (no tissue) counts to determine the effect of the tissue colour, yielding *correction factors* (correction factor = standard cpm / tissue cpm). In each biodistribution experiment, counts underestimated due to colourization of the tissues can be compensated by multiplying the tissue counts by the correction factors.

2.4 Ex Vivo Evaluation of (S)-[^{3}H]CGP12177 Binding

2.4.1 Specific Binding to βARs

To assess the specific binding of (S)-[^{3}H]CGP12177 to βARs, saturating doses of β-blockers propranolol (10 mg/kg, ip) or unlabelled (S)-CGP12177 (10 mg/kg, ip) (Law, 1993) were administered to the rats 15 minutes prior to tracer injection and biodistribution procedure performed as described in section 2.3.

2.4.2 βAR Downregulation

To determine the effect of persistent agonist stimulation of βARs on tracer uptake, rats were subjected to a 14-day administration of isoproterenol (12 mg/kg/day) through osmotic mini-pumps (Alzet) (Brouri et al., 2002). Mini-pumps that dispensed saline (0.9% NaCl) or isoproterenol were primed at 37 °C for 24 hrs and implanted according to manufacturer's instructions. Briefly, rats were anesthetised with 1-2 mL/min isoflurane, minipumps implanted subcutaneously through a lateral incision in the intrascapular area and wounds closed with surgical staples. Buprenorphine was used for pre- and post-surgical analgesia, as per standard practices of the Animal Care Committee of University of Ottawa. At the end of the 14 day infusion and following a 24 hours wash out period, a biodistribution study was performed as described above. To calculate the specific binding of the tracer, non-specific binding was measured by administration of cold (S)-CGP12177, 15 minutes prior to tracer injection.

2.4.3 The Effect of HFD and Overnight Fasting

To determine whether diet composition can affect the uptake of (S)-[^{3}H]CGP12177, Sprague-Dawley rats were divided into two groups and fed either HFD or chow diet for 56 days. Animals were then fasted overnight prior to the biodistribution study. To assess any effect that acute fasting may have on tracer uptake, the biodistribution studies were performed on Sprague-Dawley rats fed HFD for 10 days and either fed or fasted overnight.

2.5 HFD STZ-Treated Animals

2.5.1 STZ Administration

Normal Sprague-Dawley rats were fed HFD for 14 days. Following this period of high fat consumption, animals were administered a single moderate dose of STZ (45 mg/kg, ip, dissolved in 0.1 M sodium citrate buffer) or vehicle alone (control rats) (Zhang et al., 2003). To assess disease state at two different time points, rats were divided into two groups and biodistribution experiments were performed at either 10 days or 56 days following STZ-injection. Weight gain of animals was monitored throughout the experiment.

2.5.2 Blood Glucose Measurements

Blood glucose levels were measured weekly following administration of STZ. Briefly, a droplet of blood was obtained from the saphenous vein of the hind limb using a 23-gauge needle, and the glucose level was measured with a blood glucose monitor (Accuchek Inform, Roche). As described elsewhere, hyperglycaemic threshold was set as 11 mmol/L (Sawant et al., 2004; Srinivasan et al., 2005).

2.5.3 Insulin Measurements

Fasting insulin levels were assessed using a sensitive rat insulin radioimmunoassay (RIA) kit (Linco) (Jang et al., 2003). Briefly, known plasma volumes were diluted with the assay buffer (0.05 M phosphaline, pH 7.4 with 0.025 M EDTA, 0.08% Na^+ Azide, 1% RIA-grade bovine serum albumin), then incubated with a known amount of guinea pig anti-rat insulin serum and kept overnight in a refrigerator (4°C). On the second day, a

known concentration of ^{125}I-labelled insulin was added to the solution to bind to remaining unbound antibodies, and stored overnight for 24 hours at 0 °C. The final step was an addition of a precipitating reagent (goat anti-guniea pig IgG serum, 3% polyethylene glycol and 0.05% Triton X-100 in 0.05 M phosphaline, 0.025 M EDTA, 0.08% sodium azide), followed by 20 min incubation. The solutions were then centrifuged for 20 min at 3000 rpm, supernatant decanted and pellets counted in the gamma-counter to quantify the precipitated radioactivity. There is an inverse correlation between radioactivity detected and the native insulin levels in the sample, such that low radioactivity represents high analyte insulin. An insulin standard curve was created using increasing concentrations of insulin provided in the RIA kit. Calculations were performed to determine insulin concentrations (ng / mL) using the equation obtained from the standard curve.

2.5.4 Evaluation of Heart and Kidney Weights

To assess whether STZ can induce undesirable apoptotic effects on other peripheral tissues, the ratios of heart and kidney to body weight was measured in 56-day STZ-treated rats and their age-matched controls.

2.5.5 (S)-[^{3}H]CGP12177 Retention in HFD STZ-Treated Rats

To evaluate tracer retention in STZ-treated rats, biodistribution experiments were performed at 10 days or 56 days following STZ injection. Animals were fasted overnight before the biodistribution experiments, and fasting blood glucose and plasma insulin levels were obtained following sacrifice at experimental endpoints. To evaluate the

specific binding of the tracer to βARs in these animals, (S)-CGP12177 (10 mg/kg) was administered 15 minutes prior to tracer injection. Biodistribution was performed as described in section 2.2.

2.6 Data Analysis

2.6.1 Tissue Uptake of (S)-[^{3}H]CGP12177

Data analysis methods used in these studies were adapted from other groups (Van Waarde et al., 1992). According to this method, radioactivity content of the tissue is measured using Equation 1:

$$\text{Equation 1:} \quad Tissue\ uptake = \frac{cpm / g\ tissue * correction\ factor}{Total\ cpm\ injected / g\ bw}$$

Where cpm/g is the counts per minutes (radioactivity) recovered per gram of the tissue; total cpm injected is the radioactivity recovered from the standard samples, which is 0.1% of the injected dose*1000; bw represents body weight.

2.6.2 Percent Change in (S)-[^{3}H]CGP12177 Retention

Percent change in (S)-[^{3}H]CGP12177 retention in any group compared to their age-matched controls is calculated using Equation 2:

$$\text{Equation 2:} \quad Percent\ change = \frac{Tissue\ uptake_{group} - Tissue\ uptake_{control}}{Tissue\ uptake_{control}} * 100\%$$

2.6.3 Specific Retention of (S)-[^{3}H]CGP12177

To calculate the specific binding to βAR, non-specific binding was obtained by acute administration of cold (*S*)-CGP12177 in a number of rats being studied and their age-

matched controls, prior to tracer injection. βAR specific binding of the tracer is calculated using Equation 3.

Equation 3: $$Specific\ binding = Tissue\ uptake_{total} - Tissue\ uptake_{CGP12177-pretreated}$$

Where tissue $uptake_{CGP12177\text{-}pretreated}$ represents non-specific binding and tissue $uptake_{total}$ indicates total uptake of the tracer.

2.6.4 Percent Change in Specific Binding of (S)-[^{3}H]CGP12177 to βAR

Percent change in specific binding of the tracer (Equation 4) is calculated applying a combination of Equation 2 and Equation 3, where non-specific binding is subtracted from both treated and control groups.

Equation 4:

$$\%\ change\ in\ specific\ binding = \frac{specific\ binding_{group} - specific\ binding_{control}}{specific\ binding_{control}} * 100\%$$

2.6.5 Statistical Analysis

N values for each group are indicated in the corresponding Figures and Tables in results section. Statistical analyses are carried out using one-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis or with paired T-test. Significance level is set as $p<0.05$ for both tests.

3.0 RESULTS

3.1 In Vitro (S)-[^{3}H]CGP12177 Method Validation

3.1.1 (S)-[^{3}H]CGP12177 Standard Curve

The standard curve correlating counts detected using the scintillation counter with the concentration of (*S*)-[^{3}H]CGP12177 displayed a linear equation (R^2=0.9906) (Figure 3.1). The data show linearity throughout the range studied, with the lowest counts (5666.65 cpm), corresponding to the lowest concentration (0.01 μCi), and approximately 50000 cpm observed at the highest concentration (0.1 μCi).

3.1.2 The Effect of External Light on Counting Efficiency

There was no difference between detected radioactivity signals of (S)-[^{3}H]CGP12177 solutions kept overnight in light or dark conditions. Resulting counts showed overlapping lines of best fit (equations Y=468713X, Y=468214X, respectively) (Figure 3.2).

3.1.3 Tissue Radioactivity Counts of (S)-[^{3}H]CGP12177: Effect of Colour

The detectable radioactivity signals of the tracer at various concentrations were registered as significantly lower counts in the presence of the liver tissue (Figure 3.3) compared to solutions without tissue. The counts of the lowest concentration of the tracer (0.01 μCi) were registered as 5970 cpm with no tissue in solution, while the counts of the same (*S*)-[^{3}H]CGP12177 concentration were lowered to 619 cpm in presence of a highly colourized tissue (liver).

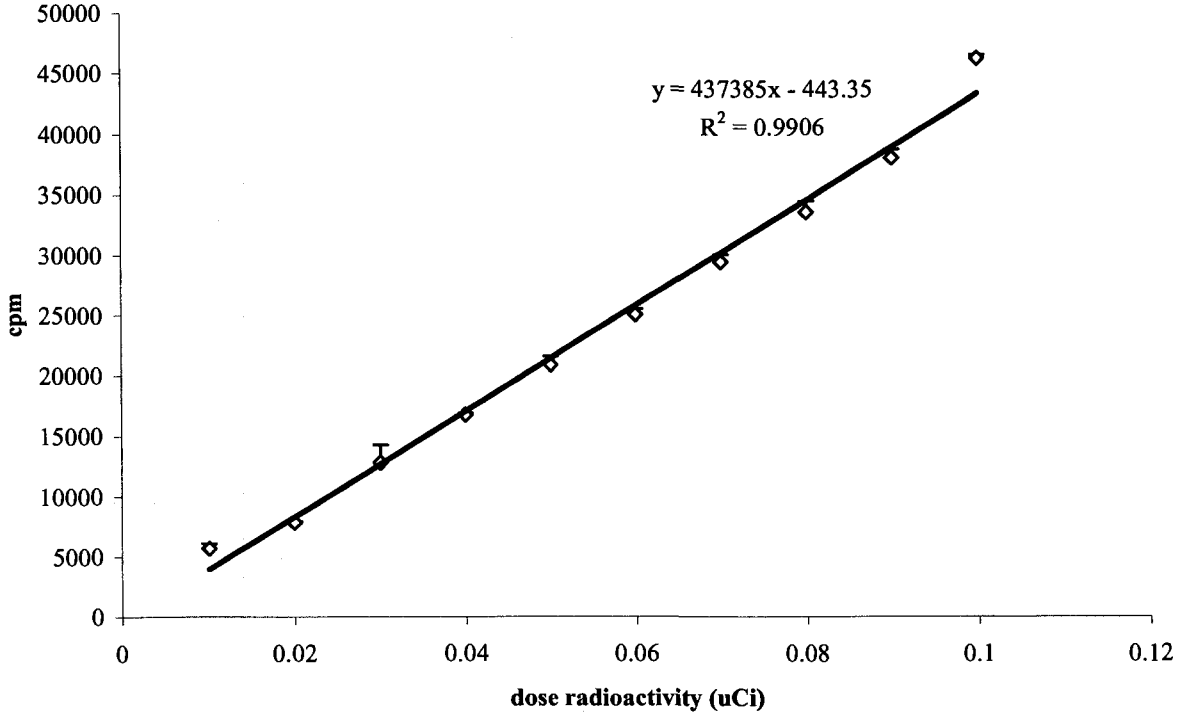


Figure 3.1: The standard curve constructed with a range of concentrations of (S)-[^{3}H]CGP12177: n=10.

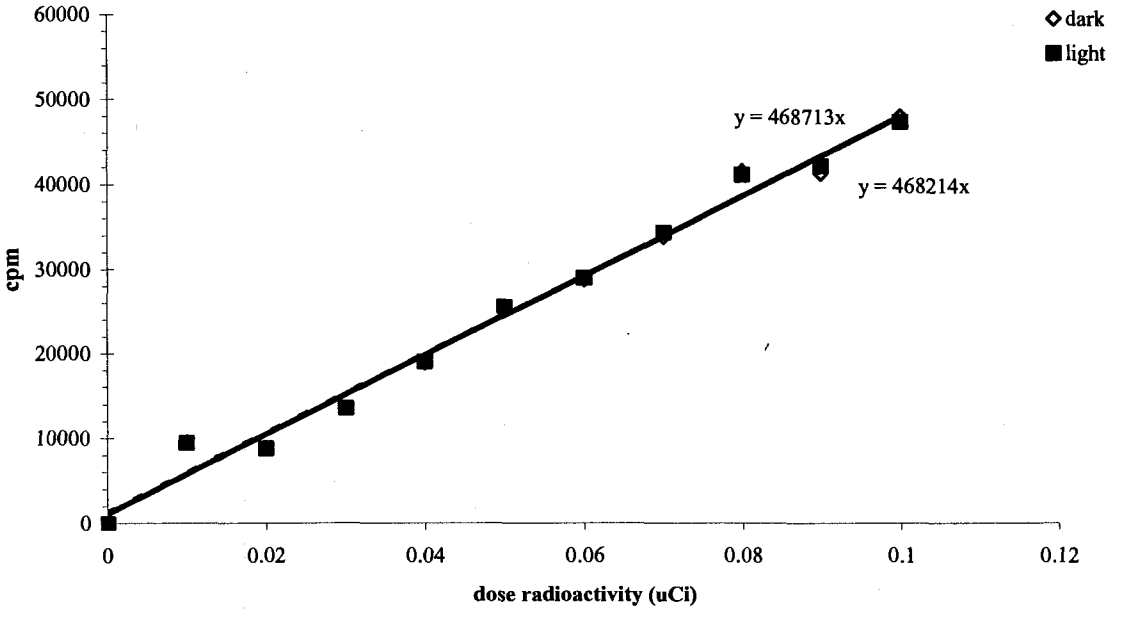


Figure 3.2: Comparison of the curves created with two series of samples left overnight in light and dark. Samples placed in the dark: n=10, samples left in the light: n=10.

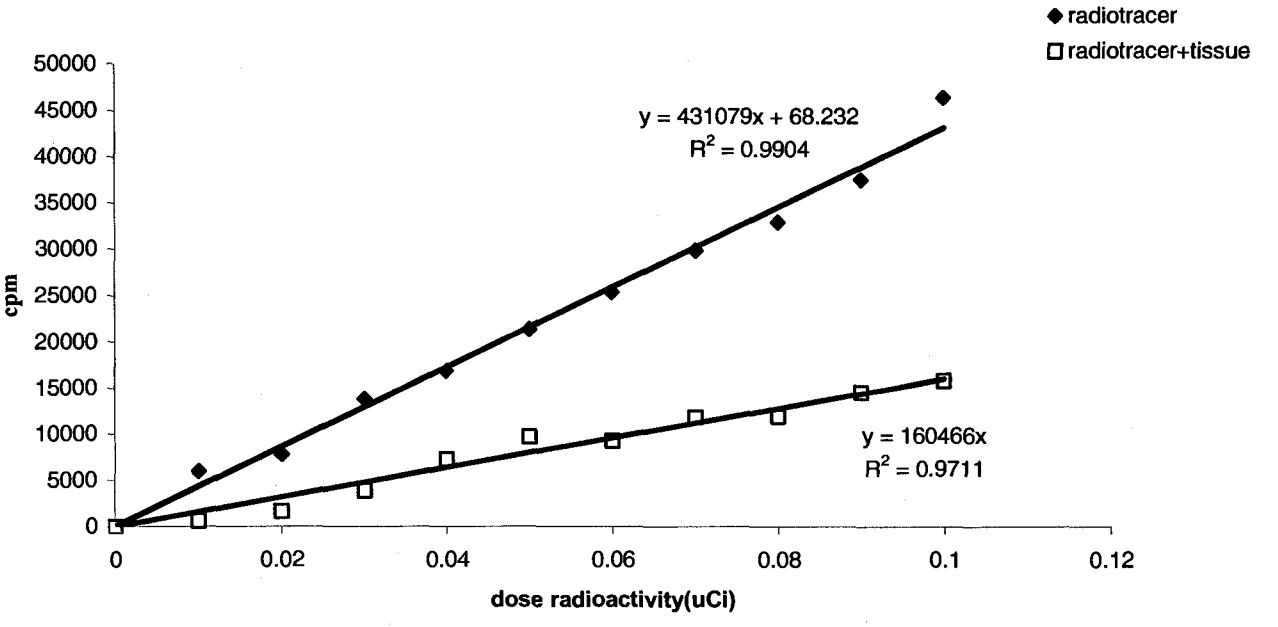


Figure 3.3: Comparison of the counts of the samples containing radiotracer alone with samples containing radiotracer and 100 mg of the tissue (liver). Samples contain tracer solution: n=10, samples contain tracer solution and tissue: n=10

The equation of the line of best fit of counts obtained with increasing dose of the radiotracer alone was found to be Y=432054X, whereas this equation was calculated as y=160466X in presence of the tissue.

3.1.4 Tissue Radioactivity Counts of (S)-[^{3}H]CGP12177: Correction Factors

By adding different tissues to a set amount of radiotracer, a range of counts was obtained (Figure 3.4). The standard sample, with 0.008 μCi added to the sample vial, exhibited greater counts (5418 cpm) compared to those of tissues. The counts of less intensely coloured tissues such as white adipose tissue and skeletal muscle were similar to the standards (4938 cpm and 4582 cpm, respectively). The lowest counts were observed in the presence of highly colourized tissues such as ventricles, the kidney and blood (3300-3600 cpm). Correction factors for different tissues were obtained by dividing the standard cpm by tissue cpm (Table 3.1).

3.2 Ex Vivo Evaluation of (S)-[^{3}H]CGP12177 Binding

3.2.1 Time Course Evaluation

The radiotracer (S)-[^{3}H]CGP12177 exhibited different uptake in various tissues at different time points (5-60 minutes). Myocardial regions exhibited high uptake at each time point compared to other tissues. The highest tracer retention in myocardial regions was observed at 30 minutes post-tracer injection, with the average uptake of 4.90 ± 0.81 (cpm/ g tissue)/(cpm inj/g bw) (Figure 3.5 A). Since highest tissue uptake was observed at 30 min post injection, this time point was selected for all subsequent ex vivo biodistribution studies.

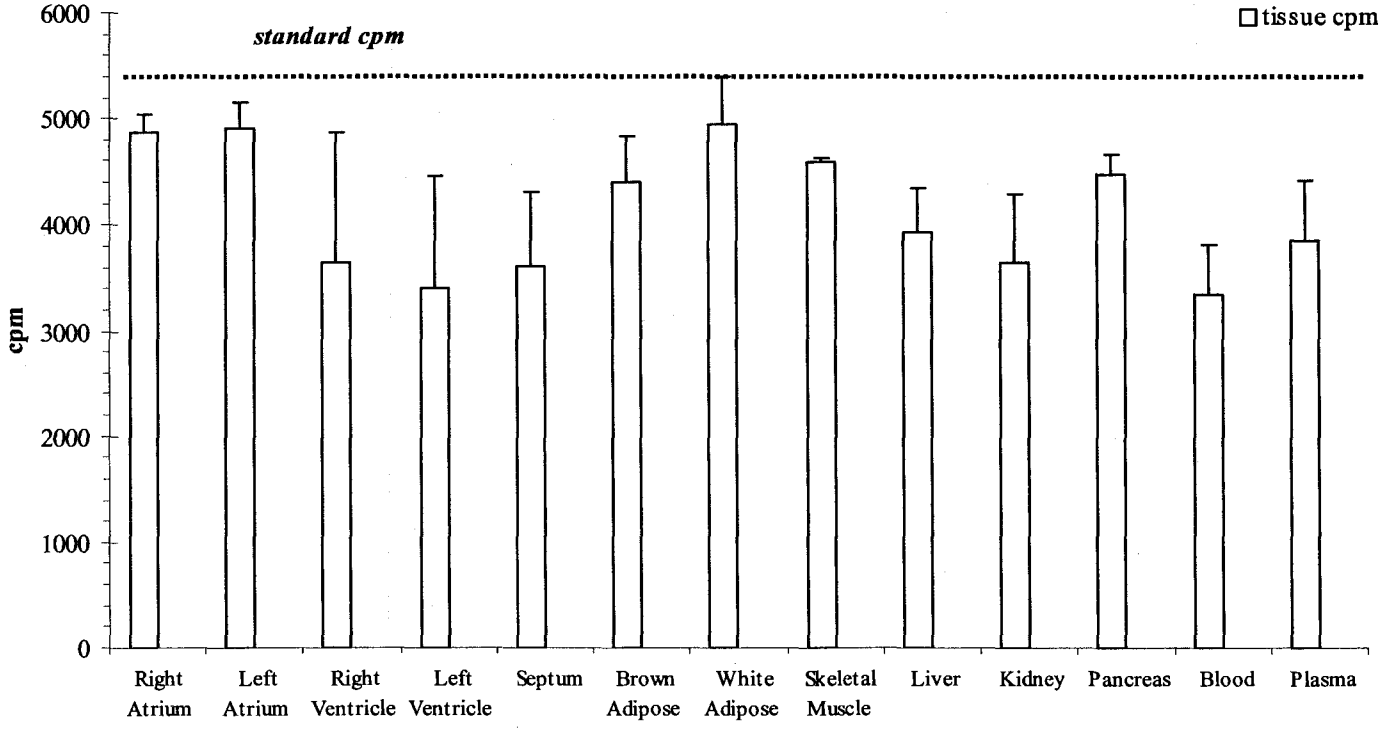


Figure 3.4: Comparison of the counts of a single dose of radiotracer (containing 0.008 μCi (S)-[^{3}H]CGP12177), in the absence (standard cpm), and in presence of various tissues (tissue cpm). Standard cpm was determined to be 5418 cpm. Standard sample: n=3, each tissue sample: n=6

* The correction factor calculated for the liver is not applicable in Figure 3.3, since the concentration of the radiotracer selected for calculations of the correction factors is 0.008 μCi, which was not used in Figure 3.3. 0.008 μCi represents 0.1% of the injected dose that is expected to be present in the tissues.

Table 3.1: Correction factors of different tissues.

Tissue	*Correction Factors*
Right Atrium	1.11
Left Atrium	1.10
Right Ventricle	1.48
Left Ventricle	1.60
Septum	1.50
Brown Adipose	1.23
White Adipose	1.09
Skeletal Muscle	1.18
Liver	1.38
Kidney	1.48
Pancreas	1.21
Blood	1.62
Plasma	1.40

Correction factors are calculated as: standard counts / tissue counts, with the standard concentration of 0.008 μCi (see Figure 3.4).

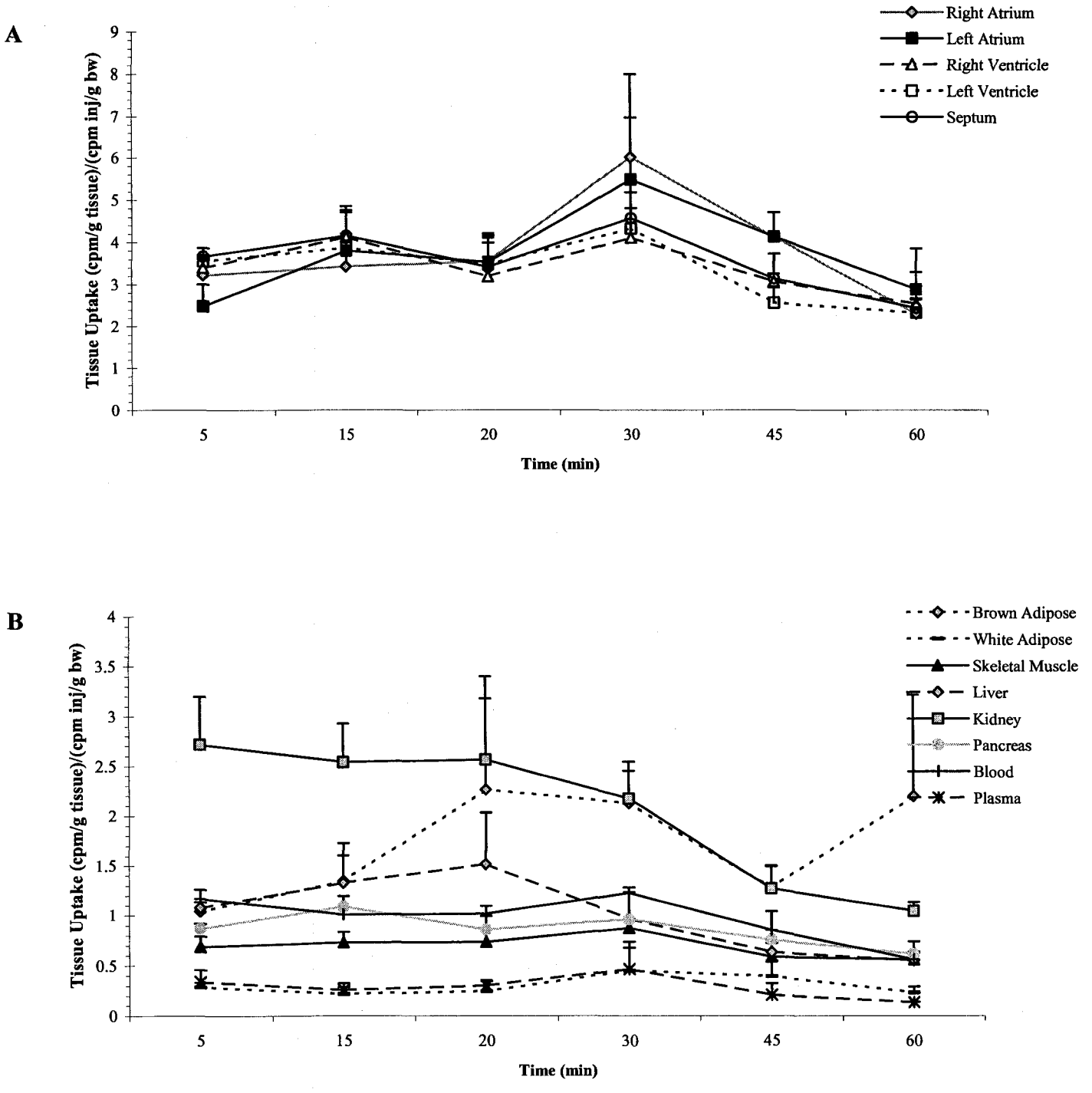


Figure 3.5: Time course of (S)-[^{3}H]CGP12177 in myocardial regions **(A),** and in other peripheral tissues **(B),** in 5-60 minutes following tracer injection. n=3 for 5, 15, 60 min, n=5 for 20 min, n=8 for 30 min, n=7 for 45 min.

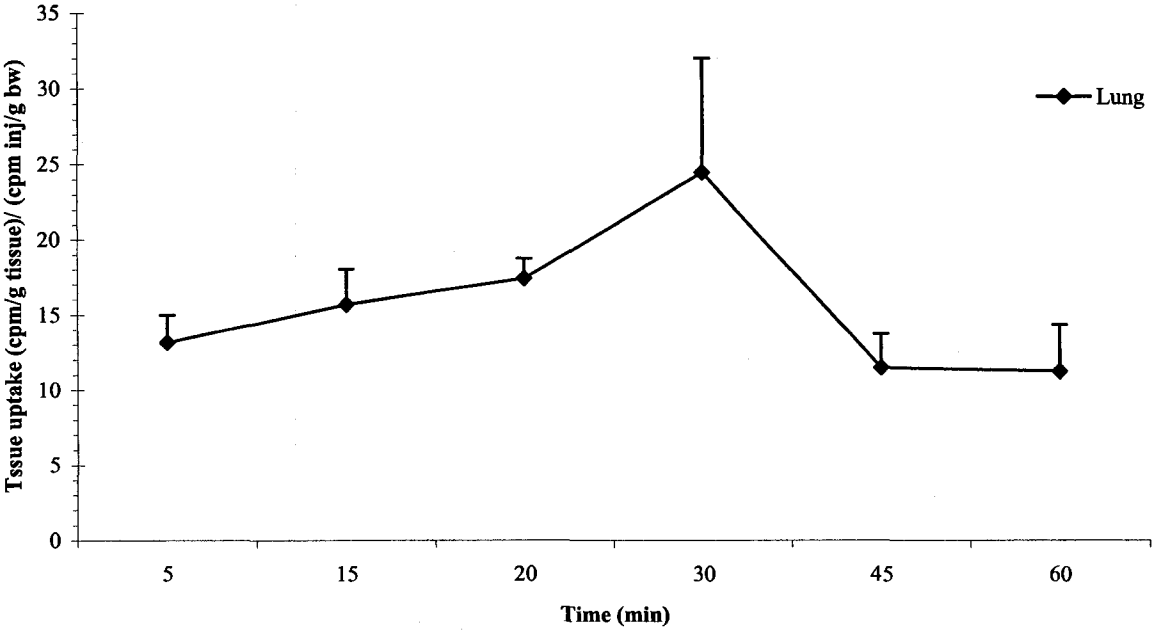


Figure 3.5.C: Time course of (S)-[^{3}H]CGP12177 in lung, 5-60 minutes following tracer injection. n=3 for 5, 15, 60 min, n=5 for 20 min, n=8 for 30 min, n=7 for 45 min.

While lower than the retention in myocardial regions, the kidney and brown adipose tissue also exhibited high tracer uptake (2.05 ± 0.71, 1.70 ± 0.54, respectively) (Figure 3.5 B). Other tissues exhibited lower uptakes (below 1) with the lowest uptake of 0.28 ± 0.11(cpm/ g tissue)/(cpm inj/g bw), observed in the plasma at the 30 min time point. Lung exhibited highest uptake with the average of 15.58 ± 5.0 (cpm/ g tissue)/(cpm inj/g bw) (Figure 3.5 C). The tracer uptake in most of the tissues remained consistent over time. Since (S)-[^{3}H]CGP12177 does not cross the blood brain barrier, the tracer uptake was not evaluated in brain regions.

3.2.2 Specific Binding to βAR

Pretreatment of animals with a saturating dose of β-blocker propranolol (10 mg/kg/day) significantly reduced tracer uptake in the myocardium (Figure 3.6). Tracer binding was reduced by 63-76% (cpm/g tissue)/(cpm inj/g bw) in myocardial regions (Table 3.2). In addition to cardiac tissues, tracer retention was significantly reduced in brown adipose tissue, skeletal muscle, kidney and blood. Tracer retention remained unchanged in white adipose tissue, pancreas and plasma. Pretreatment of the animals with unlabelled (S)-CGP12177 also significantly reduced tracer uptake in myocardium, brown adipose, kidney and blood. Total uptake after acute blocking with (S)-CGP12177 was reduced by 76-82% in myocardial regions. Table 3.2 presents percent change in tracer uptake (reflecting specific binding) in various tissues following acute pretreatment with propranolol and (S)-CGP12177.

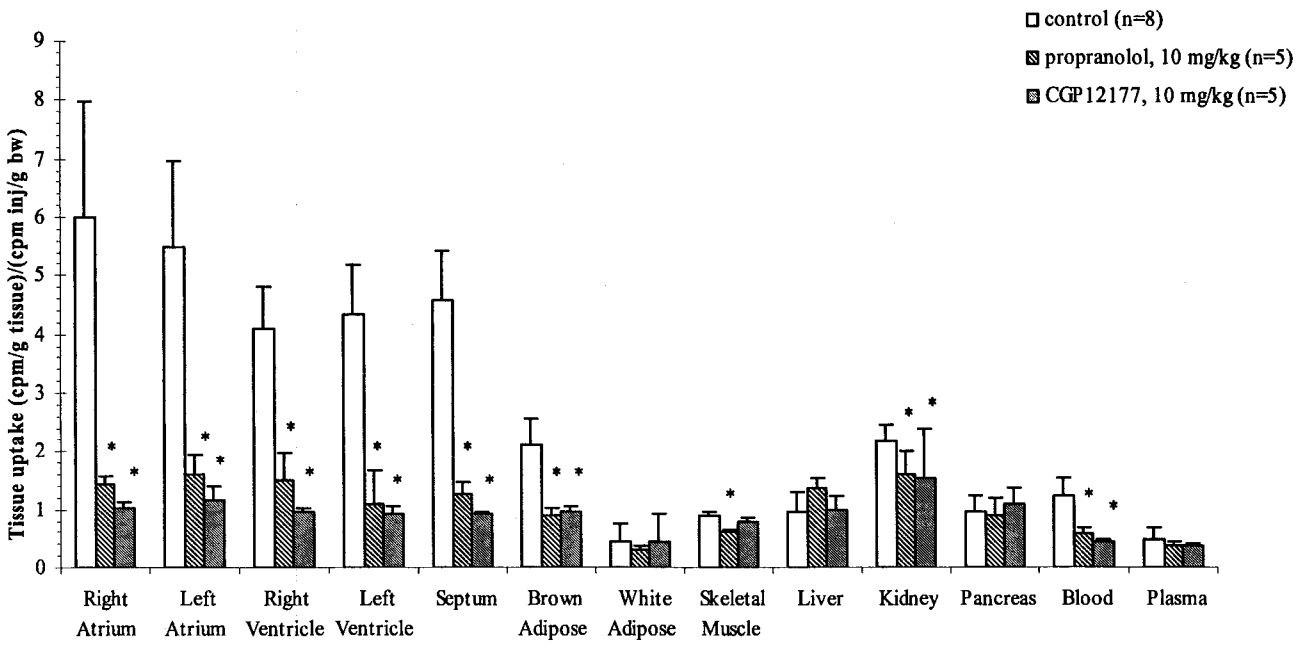


Figure 3.6: Biodistribution of (S)-[^{3}H]CGP12177 following acute pretreatment with β-blockers, propranolol (10 mg/kg, ip) and (S)-CGP12177 (10 mg/kg, ip), 15 minutes prior to tracer injection. *P<0.05 one-way ANOVA with Bonferroni post hoc compared to controls.

Table 3.2: Percent change in total (S)-[^{3}H]CGP12177 uptake in selected peripheral tissues, following acute pretreatment with β-blockers propranolol and (S)-CGP12177.

Treatment	*% change in βAR uptake of (S)-[^{3}H]CGP12177 to controls*									
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum	Brown Adipose	White Adipose	Skeletal Muscle	Pancreas	Kidney
Propranolol	-76	-70	-63	-75	-72	-58	-31	-31	-7	-26
CGP12177	-82	-78	-76	-79	-79	-55	-5	-11	13	-30

Acute pretreatments were 10 mg/kg for both propranolol and (S)-CGP12177.
Percent change to controls is calculated as (treated–controls)/(controls)*100%. *n* values are shown in Figure 3.6.

3.2.3 βAR Downregulation

The effect of 2-week administration of isoproterenol on total (*S*)-[^{3}H]CGP12177 binding was most apparent in the cardiac tissues (Figure 3.7). Total tracer uptake was reduced significantly in the left atrium, both ventricles and the intraventricular septum (41-54%) (Table 3.3). While the right atrium exhibited a trend towards reduced tracer retention (by 20%), it was not found to be significant. Similar to the myocardium, kidney tissue exhibited a significant decrease in tracer binding following sustained isoproterenol infusion. No significant alterations were observed in other peripheral tissues. (S)-CGP12177 pretreatment reduced total cardiac tracer uptake by approximately 50-72% in isoproterenol-treated and control rats (Figure 3.7). Similar to the reduction in total myocardial binding, myocardial specific binding of the tracer to βAR was reduced by 28-54% in isoproterenol-treated animals compared to controls (Table 3.4). Alterations in tracer the specific binding in the isoproterenol group in tissues of interest are shown in Table 3.4.

3.3 The Effect of Diet and Overnight Fasting

3.3.1 Effect of Acute Fasting

No difference was observed in total tracer biodistribution between animals fasted overnight immediately prior to the biodistribution studies, and the rats who had uninterrupted access to food (Figure 3.8).

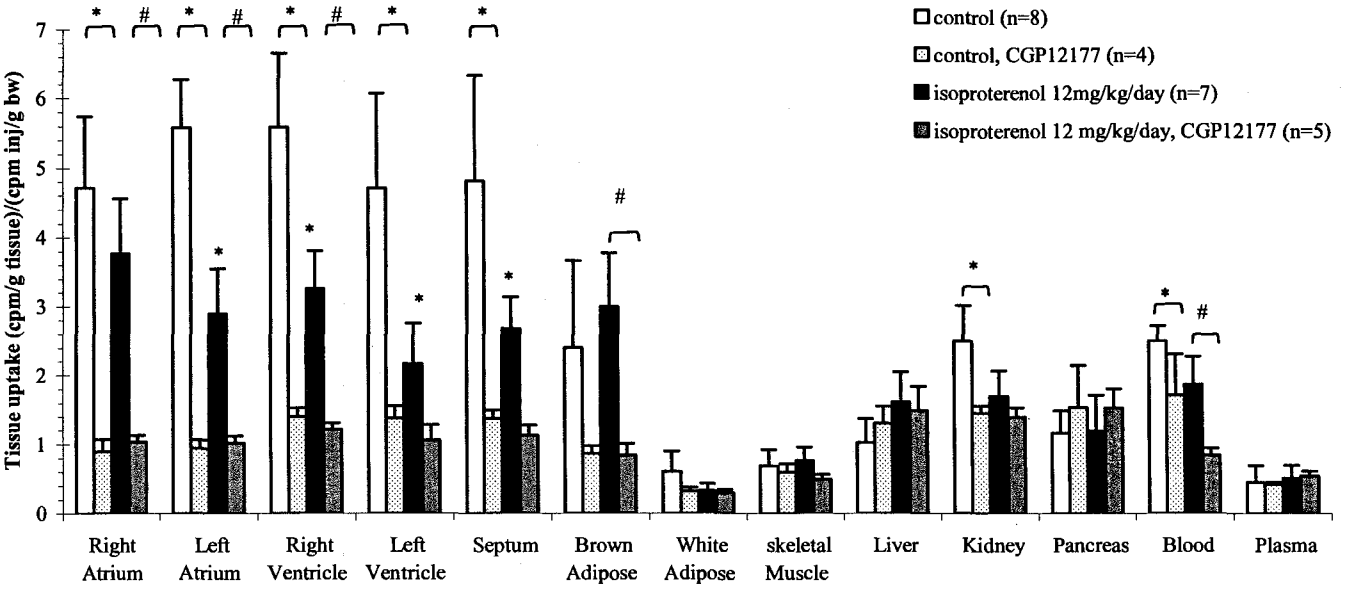


Figure 3.7: Effect of chronic stimulation of βAR with β-agonist isoproterenol (12 mg/kg/day*14 days, 24 h washout) on total (S)-[^{3}H]CGP12177 uptake. Pretreatment with (S)-CGP12177 (10 mg/kg, ip) was used to obtain non-specific uptake of the tracer in different tissues. *P<0.05 to controls, #p<0.05 to isoproterenol one-way ANOVA with Bonferroni post hoc.

Table 3.3: Percent change in total uptake of the tracer in 2-week-treated isoproterenol rats compared to the age-matched controls.

Treatment	*% change in total βAR uptake of (S)-[^{3}H]CGP12177 to age-matched controls*									
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum	Brown Adipose	White Adipose	Skeletal Muscle	Pancreas	Kidney
2-week isoproterenol	-20	-47	-41	-54	-44	69	-44	11	2	-31

2-week treatment with isoproterenol was 12 mg/kg/day*14 days with 24 hour washout period.

Percent change in total uptake of the tracer was calculated as (Tissue uptake $_{\text{isoproterenol-treated}}$–Tissue uptake $_{\text{control}}$)/(Tissue uptake $_{\text{control}}$)*100%. *n* values are shown in Figure 3.7.

Table 3.4: Percent change in specific binding of the tracer to βAR in 2-week-treated isoproterenol rats

Treatment	*% change in βAR specific uptake of (S)-[^{3}H]CGP12177 to age-matched controls*									
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum	Brown Adipose	White Adipose	Skeletal Muscle	Pancreas	Kidney
2-week isoproterenol	-28	-59	-51	-66	-54	39	-89	190	-8	-70

2-week treatment with isoproterenol was 12 mg/kg/day*14 days with 24 hour washout period. Specific binding was obtained with acute preptreatment with (S)-CGP12177. Percent change is calculated as $(Sb_{isoproterenol\text{-}treated}-Sb_{control})/(Sb_{control})*100\%$, where Sb=specific binding. *n* values are shown in Figure 3.7.

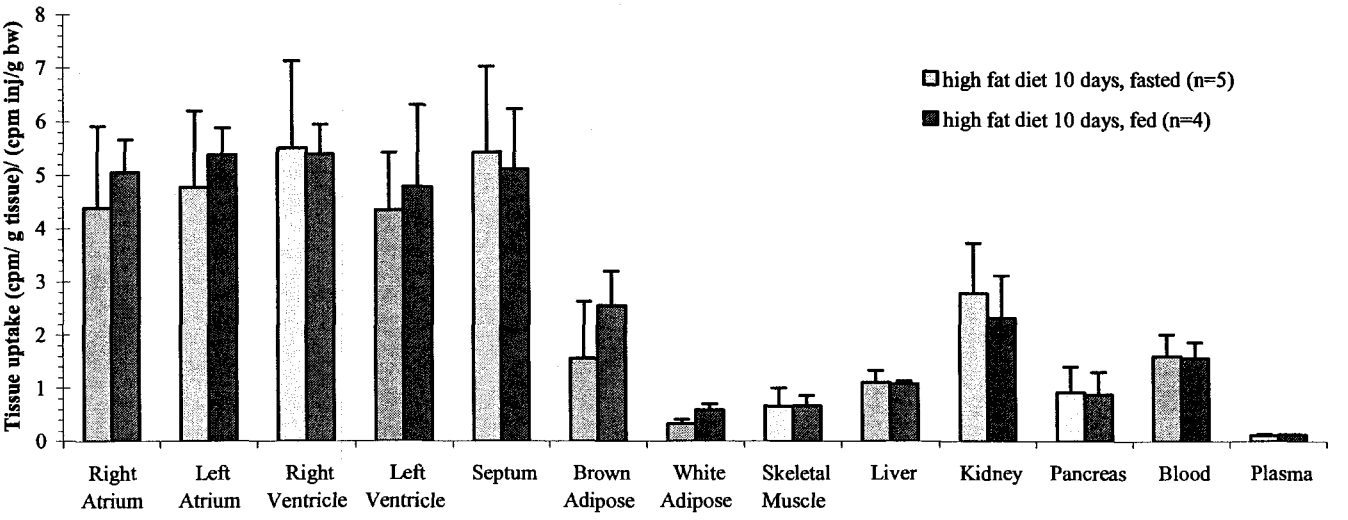


Figure 3.8: Effect of acute fasting (24 hours prior to sacrifice), 10 days following consumption of HFD on tracer uptake.

3.3.2 Effect of HFD

No significant change was observed in the total and specific tracer uptake in HFD fed group compared to chow fed rats in the myocardium (Figure 3.9 A, Table 3.5, Table 3.6). (S)-CGP12177 pretreatment reduced total cardiac tracer uptake by 74-78%, both in chow fed and HFD fed rats. In contrast, tracer retention was significantly increased in the kidney (443%) in the HFD-fed group following acute blocking with (S)-CGP12177 (Figure 3.9 B). Other peripheral tissues failed to display any significant alteration in tracer retention following acute blocking with (S)-CGP12177.

3.4 STZ-Treated HFD Rats

3.4.1 Body Weights

Following 45 mg/kg STZ injection, animals that became hyperglycaemic (blood glucose > 11 mmol/l) displayed significantly reduced weight gain compared to vehicle-treated controls. A subset of rats that maintained normal blood glucose levels following STZ injection (euglycaemics) exhibited body weight gain pattern similar to the age-matched controls (Figure 3.10).

3.4.2 Blood Glucose Levels

Following STZ injection, rats developed two distinct patterns of glycaemic control and blood glucose levels. Out of 30 STZ-treated rats, 19 rats (63%) were determined to be hyperglycaemic and 11 (36%) exhibited normal glucose levels (euglycaemic) (Figure 3.11). The glycaemic states of rats remained constant throughout the study.

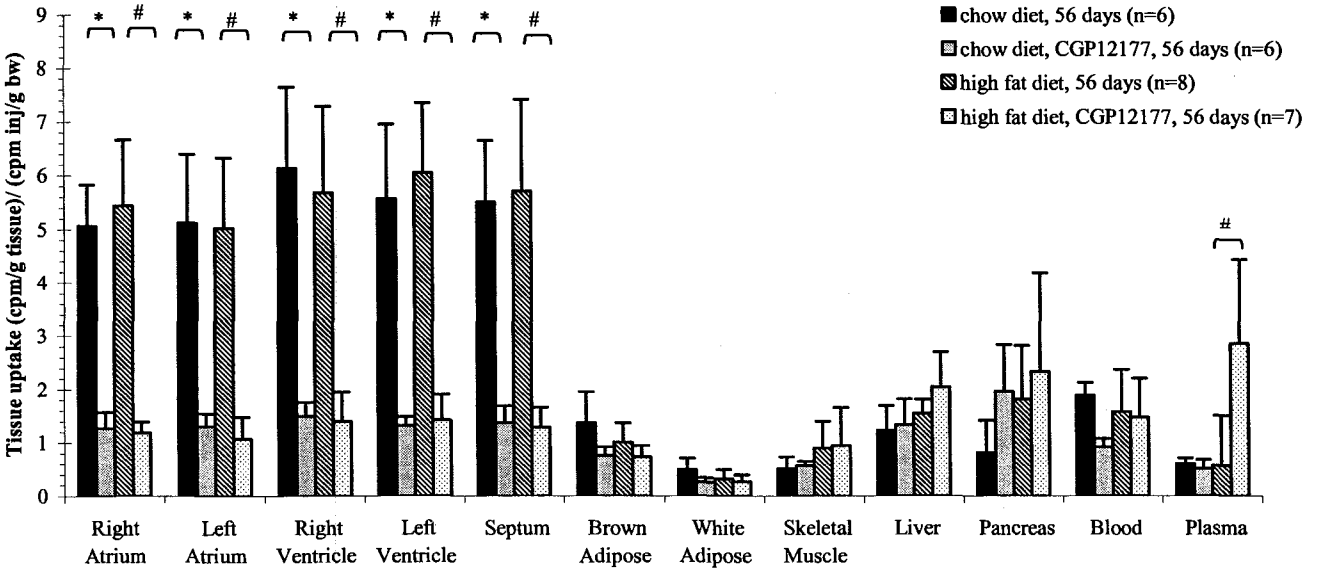


Figure 3.9 A: Comparison of biodistribution of (S)-[^{3}H]CGP12177 in chow fed and high fat fed rats, 56 days following consumption of the diets. Pretreatment with (S)-CGP12177 (10 mg/kg, ip) was used to obtain non-specific uptake of the tracer in different tissues. *$p<0.05$ to chow diet, #$p<0.05$ to high fat diet, one-way ANOVA with Bonferroni post hoc.

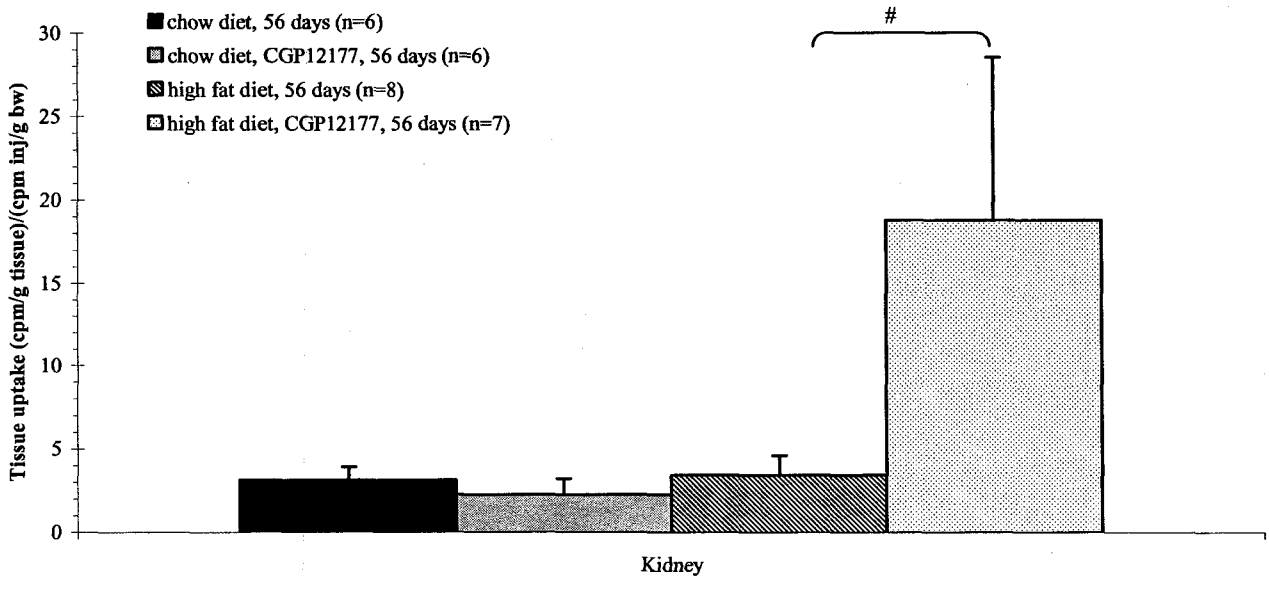


Figure 3.9 B: Comparison of biodistribution of (S)-[^{3}H]CGP12177 in chow fed and HFD fed rats in kidney, 56 days following consumption of the diets. #$p<0.05$ to high fat diet, one-way ANOVA with Bonferroni post hoc.

Table 3.5: Percent change in the total (S)-[^{3}H]CGP12177 uptake in HFD fed rats compared to chow fed rats.

Group	*% change in total uptake of (S)-[^{3}H]CGP12177 to chow fed group*									
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum	Brown Adipose	White Adipose	Skeletal Muscle	Pancreas	Kidney
HFD fed	7	-1	-7	8	3	-25	-34	74	123	9

Percent change is calculated as (Tissue uptake $_{\text{HFD fed}}$–Tissue uptake $_{\text{chow fed}}$)/(Tissue uptake $_{\text{chow fed}}$)*100%. *n* values are shown in Figure 3.9 A, B.

Table 3.6: Percent change in the specific (S)-[^{3}H]CGP12177 uptake in HFD fed rats compared to chow fed rats.

Group	*% change in specific uptake of (S)-[^{3}H]CGP12177 to chow fed group*									
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum	Brown Adipose	White Adipose	Skeletal Muscle	Pancreas	Kidney
HFD fed	12	3	-7	8	6	-54	-77	-19	-55	-1875

Acute pretreatments were 10 mg/kg for (S)-CGP12177.
Percent change is calculated as (Tissue uptake $_{\text{chow fed}}$–Tissue uptake $_{\text{HFD fed}}$)/(Tissue uptake $_{\text{chow fed}}$)*100%. *n* values are shown in Figure 3.9 A, B.

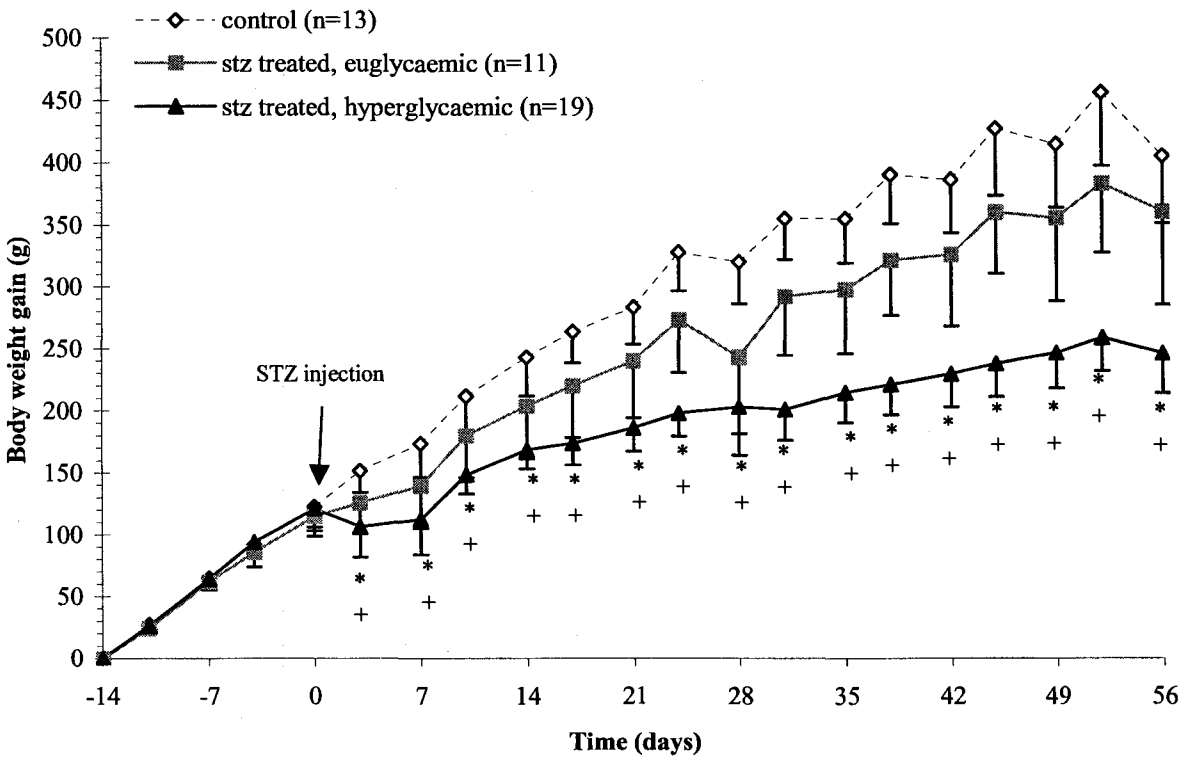


Figure 3.10: Comparison of body weight gain in STZ-treated rats with age-matched citrate-injected controls. STZ-treated rats were subdivided into euglycaemic and hyperglycaemic based on blood glucose levels. *P<0.05 to controls, +p<0.05 to euglycaemics, one-way ANOVA with Bonferroni post hoc.

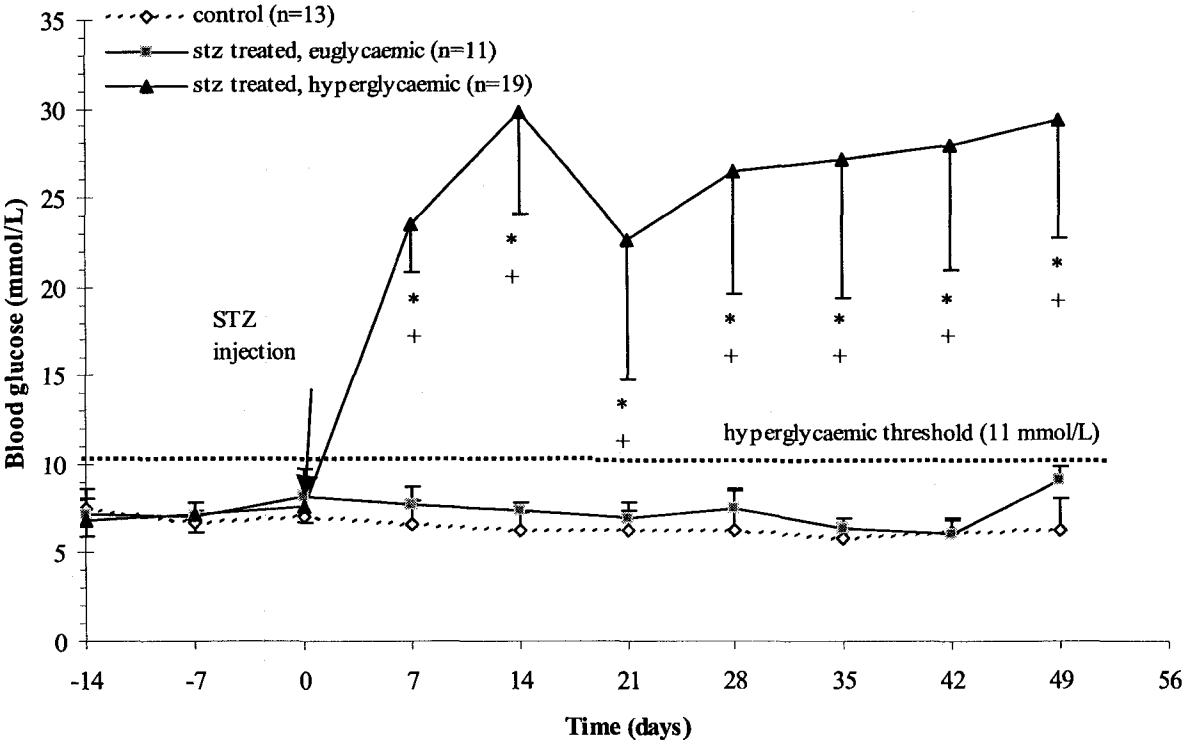


Figure 3.11: Average blood glucose levels following administration of STZ or citrate. Hyperglycaemic threshold is defined as 11 mmol/L. Animals were sacrificed and biodistribution studies were performed at 10 days or 56 days following STZ injection. *P<0.05 to controls, +p<0.05 to euglycaemics, one-way ANOVA with Bonferroni post hoc.

Fasting blood glucose levels reached approximately normal values (<11 mmol/L) in both 10-day and 56-day hyperglycaemic rats, with 56-day group showing stronger deviation from the normal levels (Table 3.7 A, B).

3.4.3 Plasma Insulin Levels

Fasting plasma insulin levels did not differ significantly between euglycaemic and control animals, but were significantly lower in hyperglycaemic rats compared to controls in 10-day rats (Table 3.8). Chronic hyperglycaemic rats (56-day) displayed a significant decrease in insulin levels compared to both control and euglycaemic groups.

3.4.4 Heart and Kidney Weights

No difference was observed in the heart weights as well as in the ratios of heart to body weights between control, euglycaemic, and hyperglycaemic rats (Table 3.9). The kidney weights and the ratio of the kidney to body weights were significantly higher in hyperglycaemic rats compared to controls. This ratio remained normal in euglycaemic rats.

3.4.5 Tracer Uptake in 10-Day STZ-Treated Rats

No difference was observed in tracer biodistribution between control, euglycaemic and hyperglycaemic rats, 10 days following STZ injection (Figure 3.12). Percent change in radiotracer retention in euglycaemic and hyperglycaemic rats compared to controls is shown in Table 3.10.

Table 3.7: Comparison of average blood glucose levels between control, euglycaemic and hyperglycaemic rats at different time points, starting 14 days prior to injection of STZ, in 10-day **(A)** and 56-day **(B)** groups.

A

Group	*Blood glucose levels (mmol/l)*			
	d_{-14}	d_0	d_7	fast at d_{10}
control	7.0±1.1	7.0±1.6	6.5±1.4	4.3±0.5
10-day, euglycamiec	7.2±0.7	6.3±0.8	7.6±1.8	5.3±2.0
10-day, hyperglycaemic	7.3±1.1	6.6±0.9	23.2±2.8*#	7.5±1.2*#

B

Group	*Blood glucose levels (mmol/l)*			
	d_{-14}	d_0	d_7	fast at d_{56}
control	7.5±0.9	7.0±0.9	6.8±1.0	3.5±0.8
56-day, euglycaemic	7.2±1.1	7.5±1.2	9.0±3.0	3.4±0.6
56-day, hyperglycaemic	6.9±0.9	8.0±1.3	23.8±5.8*#	10.0±4.0*#

n values are shown in Figure 3.11. *$p<0.05$ to controls, #$p<0.05$ to euglycaemics, one-way ANOVA with Bonferroni post hoc.

Table 3.8: Comparison of fasting insulin levels between control, euglycaemics, and hyperglycaemic rats in 10-day and 56-day post-STZ injection.

Group	*Insulin levels (ng/ml)*	
	10-day STZ	56-day STZ
control	1.15±0.08	1.28±0.14
euglycaemic	0.92±0.63	1.12±0.43
hyperglycaemic	0.84±0.26*	0.57±0.14*#

*P<0.0.5 with T-test to controls, #P<0.05 with T-test to euglycaemics. 10-day control: n=5; 10-day euglycaemic: n=4; 10-day hyperglycaemic: n=6, 56-day control: n=13, 56-day euglycaemic: n=13, 56-day hyperglycaemic: n=15.

Table 3.9: Comparison of the heart and kidney weights and the ratios of heart and kidney to body weights between control, euglycaemic and hyperglycaemic groups at 56 days post-STZ injection.

Group	***Heart Weight (g)***	***Kidney Weight (g)***	***Heart/BW(*1000)***	***Kidney/BW(*1000)***
control	1.86±0.20	1.84±0.14	3.26±0.50	3.20±0.40
56-day, euglycamiec	1.46±0.15	1.56±0.23	2.80±0.30	2.95±0.20
56-day, hyperglycaemic	1.54±0.15	2.66±0.70*	3.80±0.50	6.6±1.80*

*P<0.05 one-way ANOVA with Bonferroni post hoc compared to control. Control: n=8, euglycaemic: n=5, hyperglycaemic: n=5.

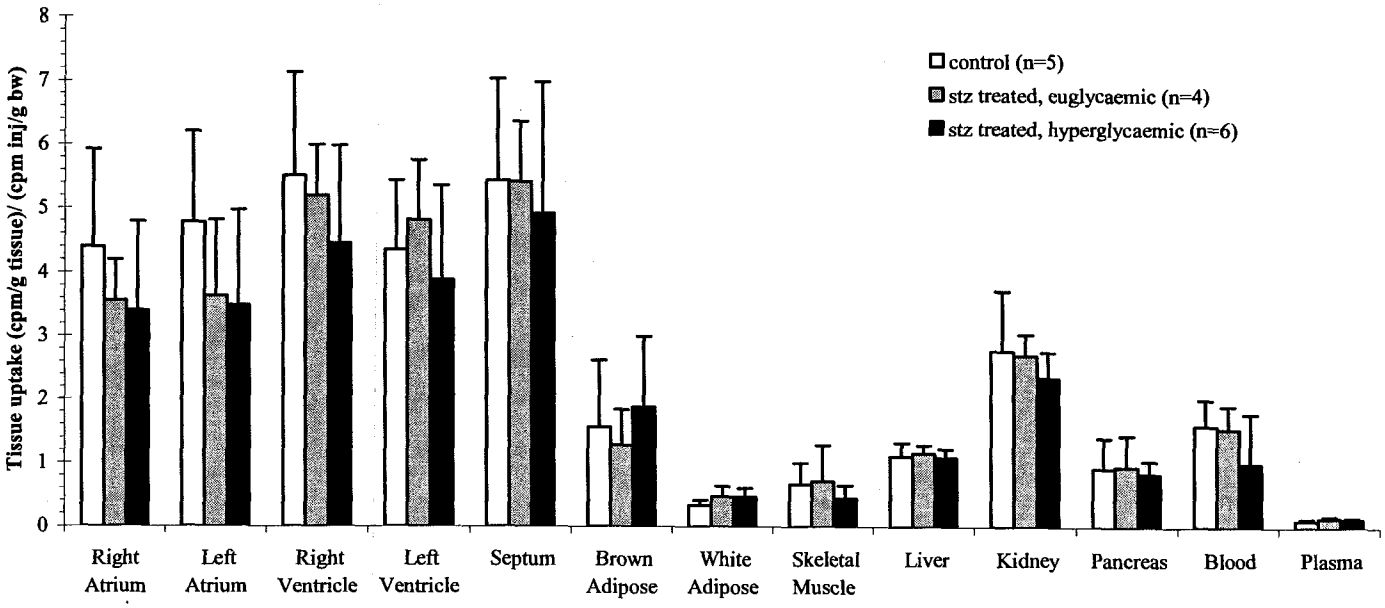


Figure 3.12: Biodistribution of (S)-[^{3}H]CGP12177 in euglycaemic and hyperglycaemic STZ-treated rats, 10 days following STZ administration.

Table3.10: Percent change in total (S)-[^{3}H]CGP12177 uptake in 10-days STZ-treated rats compared to controls.

Group	*% change in total (S)-[^{3}H]CGP12177 uptake to controls*									
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum	Brown Adipose	White Adipose	Skeletal Muscle	Pancreas	Kidney
10-day, euglycaemic	-18	-23	-5	10	-0.40	-17	41	8	3	-2
10-day, hyperglycaemic	-22	-27	-19	-10	-9	20	40	-31	-8	-15

Percent change to controls is calculated as (Tissue uptake$_{\text{STZ-treated}}$–Tissue uptake$_{\text{controls}}$)/(Tissue uptake$_{\text{controls}}$)*100%. *n* values are shown in Figure 3.12.

3.4.6 Tracer Uptake 56 Days Post-STZ Treatment

Tracer retention 56 days following injection of STZ displayed a significant reduction in cardiac regions of hyperglycaemic rats compared to controls (35-38 %) (Figure 3.13 A, Table 3.11), while no alteration was observed in euglycaemic animals. Alterations in tracer retention in other peripheral tissues are not significant in different STZ-treated groups as compared to controls. Specific binding of the tracer exhibited a 35-43% reduction in myocardium in hyperglycaemics compared to controls (Figure 3.13 A, Table 3.11). A similar percent reduction was found in total uptake of the tracer compared to controls. Kidneys exhibited a significant elevation of tracer accumulation following acute pretreatment with (S)-CGP12177 in all groups (Figure 3.13.B, Table 3.12).

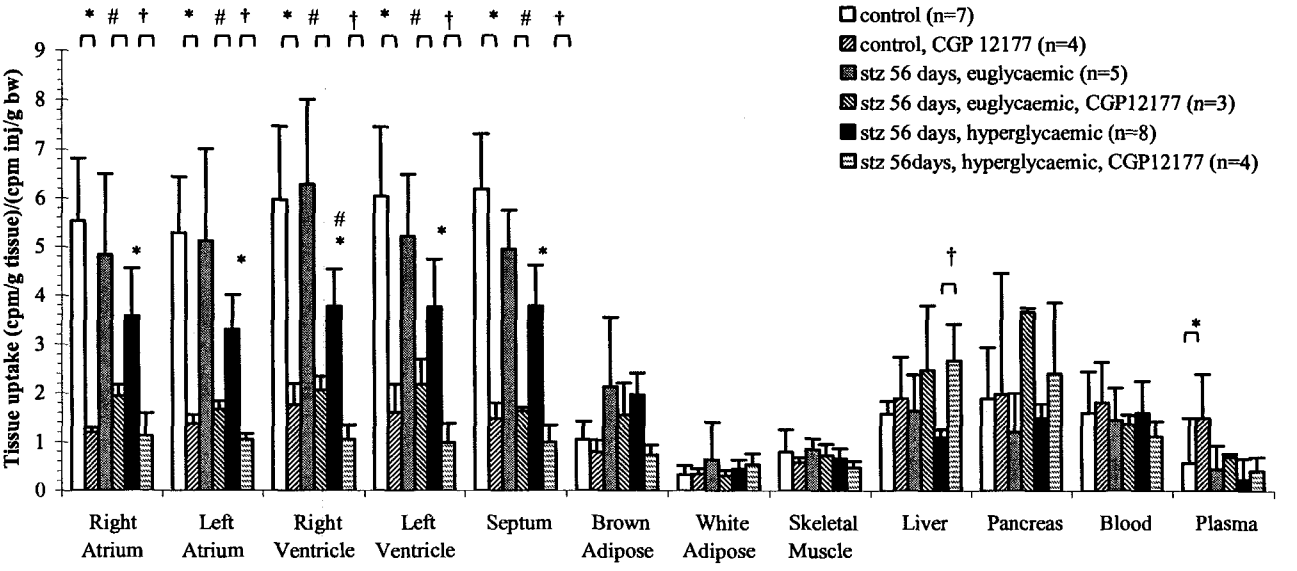


Figure 3.13 A: Biodistribution of (S)-[^{3}H]CGP12177 in control, euglycaemic and hyperglycaemic STZ-treated rats, 56 days following induction of STZ. Pretreatment with (S)-CGP12177 (10 mg/kg, 15 min prior) was used to obtain non-specific uptake of the tracer. *P<0.05 to controls, #p<0.05 to euglycaemic, †p<0.05 to hyperglycaemics, one-way ANOVA with Bonferroni post hoc.

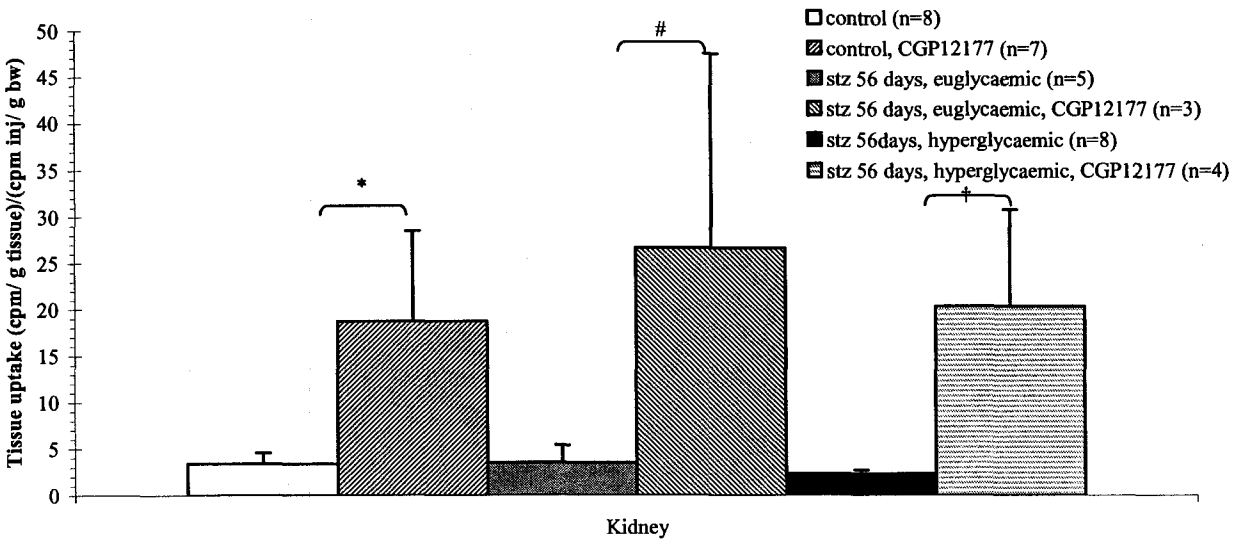


Figure 3.13 B: Comparison of biodistribution of (S)-[^{3}H]CGP12177 in control, eulgycaemic and hyperglycaemic STZ-treated rats in the kidney, 56 days following induction of STZ. Pretreatment with (S)-CGP12177 (10 mg/kg, ip) was used to obtain non-specific uptake of the tracer. *P<0.05 to controls, #p<0.05 to euglycaemic, †p<0.05 to hyperglycaemics, one-way ANOVA with Bonferroni post hoc.

Table 3.11: Percent change in total uptake of the tracer in STZ-treated (euglycaemic and hyperglycaemic) as compared to their age-matched controls, at 56 days following STZ injection.

Group	*% change in βAR total uptake of (S)-[^{3}H]CGP12177 to age-matched controls*									
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum	Brown Adipose	White Adipose	Skeletal Muscle	Pancreas	Kidney
56-day, euglycaemic	-12	-3	5	-13	-19	127	95	7	-3	3
56-day, hyperglycaemic	-35	-37	-36	-37	-38	88	40	-16	-35	-29

Percent change in total uptake of the tracer is calculated as (Tissue uptake$_{STZ\text{-}treated}$–Tissue uptake$_{control}$)/(Tissue uptake$_{control}$)*100%. *n* values are shown in Figure 3.13 A, B.

Table 3.12: Percent change in specific βAR uptake of the tracer in 56-day STZ-treated (euglycaemic and hyperglycaemic).

Group	*% change in βAR specific uptake of (S)-[^{3}H]CGP12177 to age-matched controls*									
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum	Brown Adipose	White Adipose	Skeletal Muscle	Pancreas	Kidney
56-day, euglycaemic	-33	-11	0.20	-31	-29	-153	-213	-43	2502	50
56-day, hyperglycaemic	-43	-42	-35	-37	-40	398	368	-15	865	17

Specific binding was obtained by acute pre-treatment with (s)-CGP12177.
Percent change is calculated as $(Sb_{STZ\text{-}treated}-Sb_{control})/(Sb_{control})*100\%$, where Sb=specific binding. *n* values are shown in Figure 3.13 B, C.

* The massive increase in percent change of the specific binding calculated for the pancreas in not meaningful as it is due to the low amounts of the tracer retention and lack of specific binding in this region.

4.0 DISCUSSION

4.1 Optimization of Methods Used for Determining Radioactivity Levels in Tissues

4.1.1 Principles of Liquid Scintillation Counting

The tritiated form of CGP12177 has been used in this project to measure in vivo the βAR density. Tritium, ^{3}H, is a radionuclide that emits low-energy β^- particle (0.01859 MeV). Liquid scintillation (LS) counting is the approach to count low-energy β-emissions (Bransome, 1973). The principal of the LS counting includes the incorporation of the radiolabelled analyte into uniform distribution with a liquid medium capable of converting the kinetic energy of the nuclear emission into light energy. The liquid medium or the scintillation fluid consists of various components including the solvent, each possessing a specific task in the process of scintillation. Scintillation initiates with the emission of β^- particle from the analyte in a radioactive decay. The β^- particle travels only short distances before all its kinetic energy is dissipated. The kinetic energy dissipated from the β^- particle transfers to the solvent molecules of the medium making them excited. The energy of the excited molecules is emitted as UV light upon return to the ground state. The UV light is further absorbed by another component of the medium and is emitted as blue light flashes. The light produced from the excitation of the medium, directs toward the photomultiplier tubes (PMT). Then the PMT, in turn, convert the energy from the form of blue light into the measurable electrical pulses (Figure 4.1). Nuclear decay events produce approximately 10 photons per keV of energy. The total number of the photons emitted constitutes the scintillation, and the intensity of the light is proportional to the initial energy of the β particles.

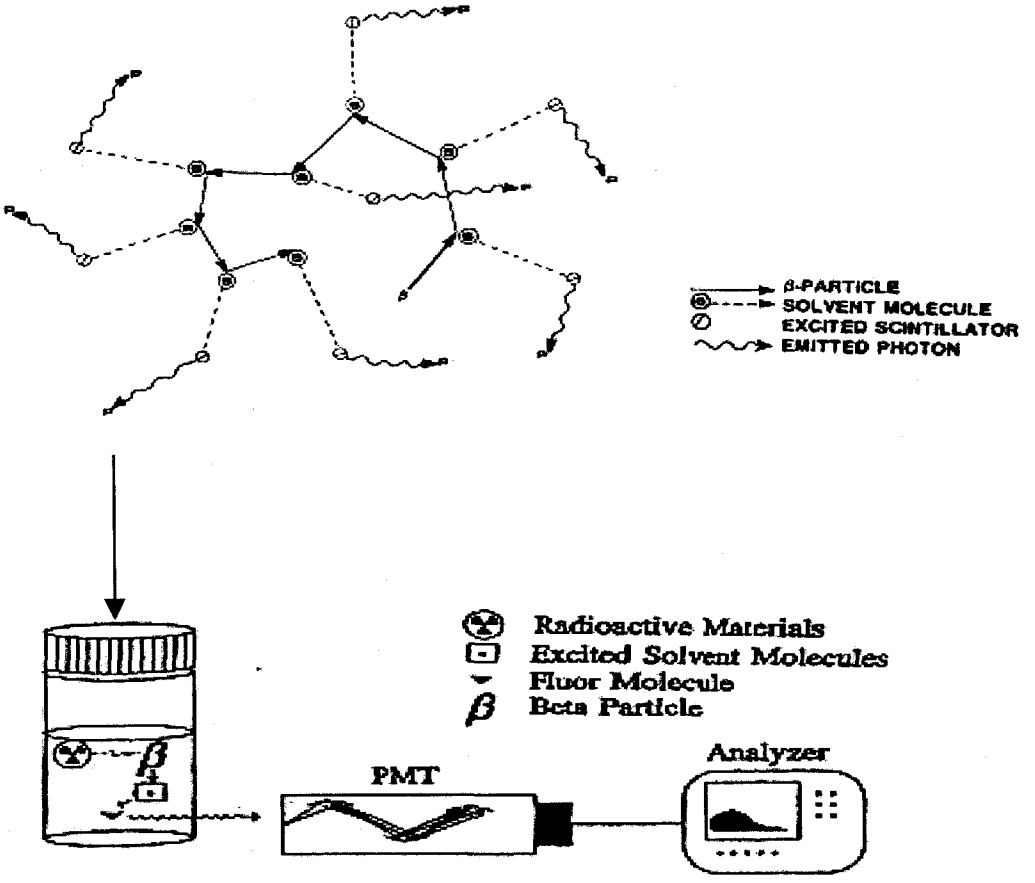


Figure 4.1: Graphic illustration of scintillation process. Emitted radiation interacts with the scintillation fluid, ultimately leading to a count which is recorded by the system. Adapted from radiation safety program manual, University of Wisconsin.

4.1.2 In Vitro (S)-[^{3}H]CGP12177 Method Validation

A standard curve of (S)-[^{3}H]CGP12177 was constructed to relate counts registered in the scintillation counter to the radioactivity dose. Various doses of the radiotracer were selected based on the estimated amounts of the radiotracer distributed throughout the body and retained by each tissue following injection of radiotracer to animals. For instance, high percent uptake of the injected dose was predicted in the lung due to the presence of high amounts of βARs. A direct correlation between cpm and dose of radioactivity was apparent, that higher doses yielded higher counts. The linear slope further implicates that LS counter is highly efficient in the counting and the counts are not dampened at the doses expected to be counted in the experiments.

Since decay events take place at different time intervals, photons reach PMTs at different times. Hence, counts from a certain dose of radioactivity were examined and monitored at various time points, and 240 seconds was established as the best time that allow more accurate measurements of radioactivity.

Photoluminescence defined as random single photon emissions due to excitation of the cocktail or vial by UV light (exposure to sunlight or UV lights) may be problematic and result in false counting. As such, to assess the influence of external light on counting efficiency, the radioactive samples were exposed to light for 12 hours. According to our results, no change was observed in counting the two sets of samples containing the same amounts of radioactivities when one placed in the dark and the other exposed to light for 12 hours. However, to eliminate any possible intrusion of light all the samples were kept in the cabinet for all the experiments.

4.1.3 Processing Tissue Samples for Liquid Scintillation Counting

The methodology developed to process the tissues for LS counting had been previously described by other centers (van Waarde et al., 1992). We have further adapted these methods to optimize the counts obtained in each tissue. To solubilize the tissues, 1 ml solubilizer was added to 100 mg of the tissue followed by 4 hours incubation. Utilizing larger amounts of the tissue such as 150 mg amplified the time of incubation. Therefore, to avoid elongation of the procedure, 100 mg was established as a maximum tissue sample to dissect. Following incubation, H2O2 was added for bleaching, and isopropranolol was further applied to prevent the foaming caused by H2O2. It is believed that solubilizer and bleaching agents present in the scintillation solution are the factors that may reduce the counting efficiency. However, since the tissues containing radioactivity should be uniformly distributed and mixed with the scintillation fluid, the use of the solubilizer seems to be inevitable. Moreover, to overcome the interference of the colour in counting, the use of decolourizing agents was also inevitable. Various amounts of H2O2 and isopropranolol were examined (0.2ml, 0.4 ml, 0.6 ml, 0.8 ml), and 0.4 ml was found to be the proper amount for both H2O2 and isopropanol, reducing the colour without causing chemiluminescence. Chemiluminescence is defined as random single photon events registered as counts as a result of interaction of the sample components. Specifically, by using high doses of decolourizing agents (>0.4 ml), chemiluminescence can be troublesome. To overcome any possible undesirable effects of chemiluminescence, a small amount of glacial acetic acid was applied to buffer the solution and eliminate any possible interaction of the chemicals.

4.1.4 Tissue Radioactivity Counts of (S)-[^{3}H]CGP12177: Effect of Colour

Anything that interferes with the processes leading to the production of light can dampen the scintillation intensity and reduce the counting. It is believed that colourization of the samples is one of the major burdens to measure the index of radioactivity, as it can severely dampen the counts. Green, red, and yellow light can absorb the blue light emitted by the medium, preventing it to reach the PMTs, and resulting in incomplete transfer of energy (Radiation Safety Manual-XIX Appendices). It seems that our trials to decolourize the samples are not completely efficient, as a subset of the tissues were still colourized. Moreover, as mentioned above, utilizing higher amounts of decolourizing agents was not the preferable option due to the undesirable effects of chemiluminescence. To evaluate the degree of the impact of colour on counting efficiency, the radiotracer, in an increasing dose, was added to 10 vials containing a highly coloured tissue. A significant reduction was observed in counting the doses of radioactivities with known related cpm, in presence of the tissues. To overcome the colour quenching, an additional approach was further applied.

4.1.5 Tissue Radioactivity Counts of (S)-[^{3}H]CGP12177: Correction Factors

To assess the degree of alteration in presence of the tissue, a novel approach was applied which includes counting a certain dose of radiotracer in two different conditions; in the absence and presence of various tissues. Hence, 0.008 μCi of (S)-[^{3}H]CGP12177 was counted and monitored as the standard count. This amount was then added to various tissues. Since the tissues are differentially coloured, various counts were obtained for the same concentration. Tissues with more dense colours such as ventricles revealed less

counts, whereas tissues with lighter colours such as skeletal muscle and white adipose tissue exhibited higher counts for the same amount of radioactivity in the solution. To correct this discrepancy, the standard count was further divided by tissue counts, and a correction factor was calculated for each tissue. By multiplying the tissue counts by correction factors, the counts approximately reached real values, relative to the amount of radioactivity present in each tissue, allowing more accurate determination of tissue uptake. This approach eliminates the intrusion of colour and compensates for the underestimated counts. As such, these correction factors were employed in data analyses in each experiment.

4.2 Characteristics of (S)-[^{3}H]CGP12177

4.2.1 Low Lipophilicity

One of the major advantages of (S)-[^{3}H]CGP12177 over other antagonists to study βAR in vivo is its low lipophilicity. Several other βAR antagonists such as [^{3}H]DHA, [^{125}I]HYP, and [^{125}I]ICYP, have proven to be decent for quantification of βARs in membrane preparations (Molinoff, 1984; Stiles et al., 1984). However, in intact cells they displayed major burdens as the amount of non-specific binding increased significantly, reaching approximately more than 40% of the total binding (Pittman and Molinoff, 1980). Chemical properties of these products display high lipophilicity that increases the possibility of non-specific binding to macromolecules (Staehelin et al., 1983). Conceivably, due to its high hydrophilicity, (S)-[^{3}H]CGP12177 lacks the disadvantages of the above antagonists and appears to be an efficient and reliable ligand for specific binding to cell surface and evaluation of the receptor density in vivo.

4.2.2 Ex Vivo Biodistribution of (S)-[^{3}H]CGP12177

The tracer exhibited high uptake and low clearance at various time points in different tissues. Since the maximal uptake was observed at 30 minute post-tracer injection in myocardial regions, the biodistribution studies were conducted using this time point of sacrifice. High tracer uptake was observed in myocardial regions, lung, brown adipose tissue and kidney. The presence of high concentrations of βAR in both atria and ventricles is crucial in the regulation of heart rate, myocardial contractility, and cardiac output. An average uptake of 4.90±0.81 (cpm/g tissue)/ (cpm injected/g bw) was found in the rat heart, which was comparable to previous reports applying the same radiotracer and methodology (4.58±0.75 (cpm/g tissue)/(cpm inj/g bw) (van Waarde et al., 1992; Van Waarde et al., 1992). Although it is suggested that βARs are expressed to a greater extent in rat atria compared to the ventricles (Horinouchi et al., 2006), no significant difference in tracer uptake was observed between the heart regions.

The uptake of the tracer in brown adipocytes was less than half of that observed in myocardial regions, being the second most densely innervated tissue (2.13±0.41(cpm/g tissue)/(cpm inj/g bw). Brown adipocytes exhibited 55-58% reduction in tracer uptake following pretreatment with β-blockers, indicating high specific binding to βARs in this tissue. Brown adipocytes are major sites of thermogenesis and lipolysis in rodents through stimulation of βARs (Nedergaard and Lindberg, 1982), and any alteration in receptor density is directly accompanied by a metabolic defect in these animals (Raasmaja and York, 1988). Therefore, high amounts of tracer retention were expected in this organ. Kidney also exhibited high (S)-[^{3}H]CGP12177 uptake, however with a lower specific binding proportion compared to the heart (26-30% reduction following

acute β-blocker administration). It has been reported that βARs are expressed in distal convulated tubules, mediating Na^+ transport through stimulation of catecholamines or exogenous agonists (Gesek and White, 1997).

Although the expression of β_1- and β_3AR has been reported in white adipocytes (Germack et al., 1997), the amounts of the tracer retention observed in our experiments was low and comparable to plasma uptake of the tracer (0.45±0.28 (cpm/g tissue)/(cpm inj/g bw). This value was comparable to a previous report assessing the uptake of (S)-[^{3}H]CGP12177 in intraperitoneal fat (0.96±0.36 (cpm/g tissue)/ (cpm injected/g bw). Similarly, pancreas failed to display appreciable amounts of (S)-[^{3}H]CGP12177 uptake and did not exhibit blockade of the tracer with β-blockers, due to low expression of βAR. Innervations of SNS and pSNS fibers to the pancreas has been demonstrated (Noble and Liddle, 2005), although, high SNS innervations are not essentially indicative of high βAR density, as a combinatory stimulation of αARs and βARs mediate SNS actions in the pancreas (Vieira et al., 2004). The B_{max} values measured in the pancreas is approximately 7.50 fmol/mg (Das et al., 2006).

Blood exhibited moderate uptake of the tracer, specifically blockable with β-blockers. It has been demonstrated that the βAR antagonist ICYP is specifically retained by lymphocytes, reflecting βAR expression (Michel et al., 1986). Contrary to the blood, plasma exhibited very low amounts of tracer uptake. Studies using (S)-[^{3}H]CGP12177 have reported rapid clearance of radioactivity from plasma following tracer injection (van Waarde et al., 1992). Furthermore, the ratio of the pellet/plasma uptake of (S)-[^{3}H]CGP12177 has been reported to increase over time, representing the existence of tracer binding sites in cell pellets and absence of such binding sites in the plasma (van

Waarde et al., 1992). The low radioactivity accumulation in the brain regions (0.35-0.95 (cpm/g tissue)/(cpm inj/g bw) confirms that this tracer does not cross the blood brain barrier as previously described (van Waarde et al., 2004).

4.2.3 Specific Binding to βAR

Generally, the use of radiolabelled antagonist tracers is favoured over agonists for the determination of βAR density. It is believed that agonists bind to the receptor in high affinity state as opposed to antagonists that bind to receptors regardless of the affinity state (Terasaki and Brooker, 1978). βAR antagonist (S)-[^{3}H]CGP12177 has been extensively evaluated for in vitro quantification of βARs in various pharmacological studies. (S)- [^{3}H]CGP12177 exhibits high affinity for βARs (Staehelin and Hertel, 1983). The K_d values of (S)-[^{3}H]CGP12177 are 0.2-0.9 nM in rat cardiac homogenates (Nanoff et al., 1987; Tsuchihashi et al., 1989). Using (S)-[^{3}H]CGP12177, the B_{max} values was 271 fmol/mg in the rat heart (Saraiva et al., 2003).

Previous studies were performed to evaluate the binding selectivity of (S)-[^{3}H]CGP12177 for βARs over αARs in vivo. With this regard, administration of α-antagonists yohimbine and prazosin prior to the tracer failed to inhibit the tracer retention, suggesting that the tracer has no affinity for αARs (Van Waarde et al., 1992).

In our experiments, the high specificity of the (S)-[^{3}H]CGP12177 and its selective binding to βAR was examined through treatment of animals with unlabelled (S)-CGP12177 or propranolol, prior to the tracer injection. Other groups evaluating the specificity of the tracer using propranolol or CGP12177 administered these drugs 5 minutes prior to the tracer injection. We have selected 15 minute for the pretreatment as

this time point is more practical when injecting a large number of the rats with the cold compounds (β-blockers) in biodistribution experiments. These drugs have long biological half-lifes (Bargar et al., 1983), and are expected to be present at the receptor sites for 15 minutes. Similar to (S)-CGP12177, propranolol is also a potent selective βAR blocker that binds with high affinity to βARs. A significant decrease in tracer uptake was observed in myocardial regions following treatments with these β-blockers, confirming the specific binding of the tracer to βAR. As mentioned above, brown adipose tissue, kidney, and blood also exhibited significant tracer retention decreases (specific binding to βAR), although to a lesser extent compared to the myocardium. Previous trials have reported significant reduction in tracer uptake in the lung, and various heart regions following pretreatments with unlabelled (S)-CGP12177 and propranolol (Van Waarde et al., 1992; Law, 1993). Other peripheral tissues failed to exhibit significant reduction in tracer uptake upon pretreatment with β-blockers at 30 minutes post injection, which is in agreement with the literature (Van Waarde et al., 1992). In addition, the B_{max} values of the receptor calculated in different tissues in various studies (Snavely et al., 1982; Rothwell et al., 1985; Kompa et al., 1999) are in agreement with the amounts of the tracer uptake observed here. Accumulation of the tracer over time in the liver is due to metabolism (Law, 1993).

Pretreatment with (S)-CGP12177 resulted in greater reduction of tracer uptake as compared to the propranolol group, particularly in myocardial regions. This effect is due to the fact that (S)-[^{3}H]CGP12177 and unlabelled (S)-CGP12177 have the same structure causing the blockade of saturable non-specific binding sites, and thus resulting in greater reduction in tracer uptake compared to propranolol blockade.

4.2.3.1 Specific Binding in the Kidney

The specific binding of the tracer exhibited high variability in some regions including brown adipose tissue and the kidney in our experiments. Kidney uptake exhibited a marked increase in tracer retention following acute blocking with (S)-CGP12177 in HFD fed rats, whereas it did not display any significant change in chow fed rats. This trend was repeatedly observed in STZ-treated HFD rats (euglycaemics and hyperglycaemics) and their age-matched HFD fed controls after blocking with (S)-CGP12177. The correlation between consumption of HFD and increased radiotracer retention following blocking with (S)-CGP12177 in the kidney has still remained elusive. Some reports indicate the role of HFD on changes in physico-chemical properties of the membrane, receptor affinity, and other factors involved in cell-signalling (McMurchie et al., 1987; McMurchie and Patten, 1988). This increase in uptake is caused by non-specific binding since all the βARs are blocked with the massive dose of CGP12177. Moreover, the high radioactivity accumulation in the kidney following administration of CGP12177 may be due to intrarenal incorporation of the metabolites (less possible due to the lack of labelled metabolites in the plasma) or impaired excretions as a result of alterations in the kidney circulation or glomerular filtration rates. Further investigations are required to fully elucidate the factors underlying the variations in tracer specific binding in some regions.

4.2.4 Measuring Downregulated βAR

Subchronic treatments with β-agonist isoproterenol (K_d=15±9 nM) (Marsh and Roberts, 1987) led to a significant reduction in total and specific binding of (S)-[^{3}H]CGP12177 to βAR in the heart, indicating reduced βAR binding. In support of this finding, several

studies have reported reduced βAR numbers following chronic β-agonists treatments (Hadcock and Malbon, 1988 Hadcock, 1989 #8138; Hardin and Lima, 1999). Furthermore, these results validate the capability of (S)-[^{3}H]CGP12177 to measure reduced binding to βARs in vivo efficiently, both in stimulated and non-stimulated states.

4.2.5 Analysis of HFD, Fasting

Our results exhibit no significant difference in (S)-[^{3}H]CGP12177 uptake between chow fed and HFD fed rats. Some reports indicate the impact of dietary fatty acids and cholesterols in downregulation of βARs in cardiomyocytes (McMurchie et al., 1987; McMurchie and Patten, 1988). However, such a phenomenon was not observed in our studies. Acute fasting did not induce any marked alteration in (S)-[^{3}H]CGP12177 uptake, implicating no alteration in receptor density. Although it has been demonstrated that acute fasting alters βAR expression in brown adipocytes (Hadri et al., 1997), our results failed to exhibit any alteration in tracer uptake in any region.

4.3 STZ-Treated HFD Rat Model

4.3.1 General Characteristics of Animals

The STZ-treated hyperglycaemic rat model employed in this project had previously been used by other groups as an animal model of type II DM (Reed et al., 2000; Sawant et al., 2004; Srinivasan et al., 2005). It has been shown that a combination of HFD and moderate dose STZ produces a physiological state, which closely resembles human manifestations of type II DM. As described in detail previously, consumption of HFD induces peripheral insulin resistance accompanied by compensatory hyperinsulinemia to

maintain normal blood glucose levels. Further administration of moderate dose STZ causes partial destruction of pancreatic β-cells, resulting in progressive decline in insulin secretion, and thereby leading to hyperglycaemia (Reed et al., 2000; Sawant et al., 2004; Srinivasan et al., 2005). Glucose tolerance is impaired by HFD feeding rats and deteriorates further with moderate dose STZ (Reed et al., 2000). In addition, insulin sensitivity was shown to be decreased in STZ-treated HFD rats (Zhang et al., 2003). Our results corroborate the literature with 55-60% of STZ-treated HFD rats developing rapid hyperglycaemia, whereas the remainder maintained euglycaemia (Zhang et al., 2003; Srinivasan et al., 2005). Hyperglycaemic rats exhibited little body weight gain throughout the study, while the body weight gain pattern of euglycaemics resembled to the control group. Fasting insulin levels were significantly decreased in hyperglycaemic compared to euglycaemics rats and vehicle HFD controls. Decreased insulin levels in hyperglycaemic rats implicate reduced insulin secretion due to partial destruction of pancreatic β-cells, and matches with later stages of the disease in human. No difference was observed between euglycaemics and controls, which indicate that a moderate dose of STZ did not kill β-cells, producing similar insulin levels comparable to controls not treated with STZ.

Fasting blood glucose levels regress by 10-15 mM reaching approximately normal values in hyperglycaemic rats. This observation implies that insulin action is maintained and functional when unopposed by glucose loading but ineffective in presence of glucose, and is the hallmark factor distinguishing this model from that of high dose STZ-treated models (type I DM) (Ramanadham et al., 1983). As shown in Table 3.7, fasting glucose levels in 56-day hyperglycaemic rats are higher than those of 10-day hyperglycaemic

rats, suggesting that glycaemic control deteriorates over time (Wilkin, 2001; Newman et al., 2004). The ratios of heart to body weight were not different between control, euglycaemic and hyperglycaemics. This finding indicates that STZ does not act in the myocardium at the dose used in this study (Ramanadham et al., 1983). The different responses of animals to a moderate dose of STZ, developing two distinct glycaemic states (hyperglycaemia and euglycaemic), allow the evaluation of a single variable (hyperglycaemia) on SNS integrity, radiotracer uptake, and βAR density in these animals. The ratios of kidney to body weights were not different between euglycaemics and controls, but were significantly higher in hyperglycaemic rats. Enlargement of the kidney is one of the characteristics of the disease state (hyperglycaemia) in these animals (Bak et al., 2000). Other studies have reported increased plasma cholesterol and triglycerides in STZ-treated HFD models compared to their age-matched controls (Zhang et al., 2003; Srinivasan et al., 2005).

4.3.2 Tracer Biodistribution in STZ-Treated HFD Rat Model

A significant reduction in (S)-[^{3}H]CGP12177 uptake was observed in the myocardium of chronic hyperglycaemic rats, reflecting reduced binding to βARs. Our results strongly suggest that hyperglycaemia and/or cumulative metabolic defects of this animal model are linked to the reduced binding to the cell-surface βARs. No alteration in the tracer uptake was detected in euglycaemic rats, further corroborating the substantial impact of hyperglycaemia on alterations in SNS signalling and βAR density. Several reports support the role of glucose in SNS activation and NA release through direct effects on hypothalamic neurons or reflex responses of autonomic neurons to glucose via glucose

sensors (Oomura, 1983; Niijima, 1984; Levin and Sullivan, 1987; Levin and Sullivan, 1989; Levin et al., 1998). Accordingly, in diabetic patients, several SNS dysfunctions have been related to hyperglycaemia and other metabolic abnormalities (Pagani et al., 1988; Bellavere et al., 1992; Manzella et al., 2001; Carnethon et al., 2003). Various trials, by assessing the presynaptic fate of neurotransmitters, aimed to further evaluate the SNS function in diabetes. It is reported that NA concentration and its total spillover are enhanced in type II DM (Ferraro et al., 1993; Tack et al., 1996). Correspondingly, decreased [^{11}C] HED and [^{123}I]MIBG retention strongly suggest enhanced NA concentrations in this disease (Kiyono et al., 2002; Thackeray et al., 2006; Takahashi et al., 2007). Measurements of NA transporters support further these contentions, as reduced NA transporters impair NA reuptake, leading to increased synaptic NA levels (Kiyono et al., 2002). On the other hand, βARs are directly affected by presynaptic neuronal behaviour in normal and disease states, and the receptor density is reflective of SNS activity (Communal et al., 1998). Taken together, it was hypothesized that hyperglycaemia induces sustained SNS stimulation and NA release. Elevated NA release, in turn, produced reduced binding to βARs. Our previous work using [^{11}C]HED in the same animal model indicates reduced [^{11}C]HED retention in hyperglycaemic rats, suggesting enhanced NA concentration (Thackeray et al., 2006). This observation confirms our hypothesis that hyperglycamia is a major factor in SNS overactivity and contributes to reduced myocardial (S)-[^{3}H]CGP12177 binding to βARs (probably due to downregulation) in this animal model.

Moreover, following 10 days of STZ injection, no difference in tracer retention was observed in either STZ-treated groups compared to controls, suggesting that alteration in

tracer uptake and receptor density is due to severe glucose dysregulation and repeated effects of high glucose levels on the βARs. No significant alteration was observed in other peripheral tissues in this hyperglycaemic animal model.

In vitro binding assays for quantification of the βARs, which have been extensively performed by other groups, can be further employed in this project to measure B_{max} values of the receptor in the myocardium using (S)-[^{3}H]CGP12177. This method would also allow the evaluation of the binding affinity (K_d values) of the tracer. Moreover, in vitro measurements of the cell-surface and internalized receptors using hydrophilic ((S)-[^{3}H]CGP12177) and lipophilic ((S)-[^{3}H]DHA) ligands would provide an accurate index of the number of cell-surface and internalized receptors, further elucidating the fate of the βARs following persistent stimulation with NA in hyperglycaemic rats. Analysis of βAR mRNA level and/or evaluation of the protein expression give insights to the molecular processes underlying the reduced (S)-[^{3}H]CGP12177 retention in these animals.

4.3.3 βAR Density in Type I Diabetes

Myocardial receptor and post-receptor βAR signalling has been widely investigated in animal models of type I DM that exhibit sustained hyperglycaemia (Ingebretsen et al., 1983; Williams et al., 1983; Atkins et al., 1985; Ramanadham and Tenner, 1987; Nishio et al., 1988; Roth et al., 1995; Dincer et al., 2001). Several studies have repeatedly observed that βAR density and βAR-mediated AC activation and cAMP production are reduced in type I DM rats, while no alteration was detected in receptor affinity or AC responsiveness, suggesting that altered cAMP production is due to deficient βAR expression (Williams et al., 1983; Ramanadham and Tenner, 1987; Nishio et al., 1988;

Beenen et al., 1997). Furthermore, Williams and colleagues observed no change in muscarinic receptors in type I DM rats (Williams et al., 1983). Reduced βAR number as a result of non-enzymatic glycosylation of βARs has also been suggested in STZ-treated type I DM rats (Atkins et al., 1985). It has been reported that reduced βAR density in STZ-treated type I DM rats is reversed through glycaemic control with insulin (Ramanadham et al., 1983; Ramanadham and Tenner, 1987). This finding strongly suggests that reduced βAR density is due to several metabolic defects and concurrent SNS alterations associated with diabetes. βAR density has also been linked to hypothyroidism of diabetic state, and is reported to reach normal values with thyroxin treatment (Nishio et al., 1988).

Other studies attempted to delineate alterations in cardiac βAR signalling in type II DM. With this regard, reduced responsiveness to β-agonists and reduced cardiac contractility have been reported in both human and animal models of type II DM (Banyasz et al., 1996; Hsu et al., 1997; Joffe et al., 1999; Huisamen et al., 2001). Schaffer et al related this reduced responsiveness to agonists to attenuated AC-mediated increase in SR calcium transport. However, no change in βAR density was reported in this study (Schaffer et al., 1991). Husiamen et al investigated βAR signalling in two rat models of type II DM, obese Zucker fatty rats and neonatal-induced STZ, but observed no alterations in βAR population in either model (Huisamen et al., 2001).

5.0 CONCLUSIONS

The conclusions of this project are as follows: the radiotracer (S)-[^{3}H]CGP12177 exhibits high retention and binding specificity in myocardial regions, brown adipose tissue and the kidney. The radiotracer uptake in other tissues appeared to be low and non-specific. Moreover, (S)-[^{3}H]CGP12177 is capable of measuring reduced binding to βARs following chronic stimulation with agonist. Utilizing a combination of HFD and moderate dose STZ produced a sustained hyperglycaemic state in a short time frame, however a subset of rats maintained euglycaemia. Ten days following STZ injection, no difference in tracer uptake was observed in STZ-treated rats compared to their age-matched controls, implicating unaltered receptor density. However, at 56 days post-STZ, the radiotracer retention and specific binding to βAR displayed significant myocardial reduction in hyperglycaemics, suggesting reduced βAR binding. Compared to controls, the euglycaemic rats exhibited no alteration in (S)-[^{3}H]CGP12177 uptake at 56 days post-STZ, further confirming that high blood glucose levels was the major stimulator of SNS activity and NA release, and eventual reduction of binding to βARs in this animal model. In summary, our results demonstrate that hyperglycaemia and duration of the hyperglycaemic state are directly associated to altered SNS activity and βARs.

6.0 FUTURE DIRECTIONS

The long-term goal of this project is to evaluate βAR density in this animal model in vitro. Autoradiography experiments will be performed in myocardium of 56-day STZ-treated rats in 2008 by another student in our group. The methods used to conduct the autoradiography studies with (S)-[^{3}H]CGP12177 will be modified from our previous autoradiography experiments (Kenk et al., 2007). Briefly, the slides (4 slides per animal, 2 for total binding and 2 for non-specific binding) will be pre-washed in incubation buffer and incubated in 5nM of (S)-[^{3}H]CGP12177 for 60 minutes in 25°C. Unlabelled CGP12177 (10 μM) is used for measuring non-specific binding. The slides are then washed in the incubation buffer (4°C), dried out, and exposed to 3H-hyperfilm for 4 weeks in 4°C. The films will be developed manually (P7042, Kodak processing chemicals for autoradiography, Sigma) and analyzed using MCID™ image analysis system (Imaging Research; St Catharines, Ontario).

βAR density will be evaluated in this animal model using PET radioligands [^{11}C]CGP12177 or [^{11}C]CGP12388. Key findings will be translated to future human PET studies. Moreover, various insulin sensitizing and antihyperglycaemic drugs will be applied in this animal model. Specifically, to further elucidate the impact of glycaemia on impaired SNS signal transduction, βAR density will be evaluated before and after therapy. This would help elucidate whether glycaemic control has considerable positive outcomes in normalizing SNS activity and restoring βAR density. To further evaluate the impact of hyperglycaemia on cardiac function, echocardiographic measurements will be performed before and after therapies. As mentioned previously, CHF is 20-40% more

likely to develop in patients with diabetes than in patients without diabetes. In addition, CHF patients exhibit major burdens in metabolic controls, frequently identified by insulin resistance and impaired fasting insulin levels. The major joint factor in this cross-talk is impaired NA signal transduction, exacerbated and potentially driven by insulin resistance and hyperglycaemia. The experiments described above will also lay the groundwork for future studies evaluating comorbid CHF with type II DM in animal models, and assessing the efficacy of glycaemic control and enhanced insulin sensitivity to improve cardiac SNS function. Specifically, βAR density will be assessed in these animals applying cardiac PET imaging, biodistribution studies, and in vitro autoradiography. Therefore, this project could have potential future clinical application in guiding therapy improving cardiac function in diabetes.

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