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AGING ATTENUATES PURINERGIC, BUT NOT MUSCARINIC AND NICOTINIC, CUTANEOUS VASODILATION IN MEN

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Running head: Aging effect depends on receptor types in skin

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ABSTRACT

Objective: We evaluated the hypothesis that aging attenuates muscarinic, nicotinic, and purinergic receptor related cutaneous vasodilation.

Methods: In 11 young (24 ± 4 years) and 11 older males (61 ± 8 years), cutaneous vascular conductance (CVC) was assessed at three forearm skin sites that were infused with either: 1) methacholine (muscarinic receptor agonist, 5 doses: 0.0125, 0.25, 5, 100, 2000 mM), 2) nicotine (nicotinic receptor agonist, 5 doses: 1.2, 3.6, 11, 33, 100 mM), or 3) adenosine triphosphate (ATP) (purinergic receptor agonist, 5 doses: 0.03, 0.3, 3, 30, 300 mM). Each agonist was administered for 25 min per dose. **Results:** We showed that CVC at all doses of methacholine did not differ between groups. Similarly, no between-group differences in CVC were observed during nicotine administration at all doses administered. By contrast, while no differences in CVC were measured during the administration of ATP at low (0.03 and 0.3 mM) or high (300 mM) concentrations, CVC was reduced in the older relative to the young males at moderate concentrations of ATP (3mM: 23 ± 6 vs. $40 \pm 13\%$ max, 30 mM: 62 ± 11 vs. $83 \pm 8\%$ max, both $P \leq 0.05$).

Conclusions: We show that aging attenuates purinergic, but not muscarinic or nicotinic, cutaneous vasodilation in men.

Key words: aging, endothelium-dependent vasodilation, microcirculation

List of abbreviations: ATP, adenosine triphosphate, CVC, cutaneous vascular conductance; EC50, a concentration required to elicit a 50% maximal response

INTRODUCTION

Older adults are at greater risk of cardiovascular disease¹. In line with this, aging is associated with diminished endothelium-dependent vasodilation in conduit arteries as assessed by responses to intravascular acetylcholine infusion²⁻⁴. Alterations in the cutaneous microvasculature (accessible microvessels in humans *in vivo*) have been shown to precede overt cardiovascular disease^{5,6}. This suggests that examining this microcirculatory network may provide a sensitive indicator for age-related declines in microvascular function. However, the effects of aging on endothelium-dependent vasodilation in the cutaneous microcirculation are equivocal, such that aging has either no effect^{7,8} or it attenuates^{9,10} endothelium-dependent cutaneous vasodilation. Currently there exists a paucity of information on the influence of aging on endothelium-dependent vasodilation in the cutaneous microvasculature.

Acetylcholine can activate both muscarinic and nicotinic receptors in human skin¹¹. Methacholine, an analogue of acetylcholine, induces cutaneous vasodilation¹²⁻¹⁷ via the activation of muscarinic receptors located on endothelial cells and nicotine can induce cutaneous vasodilation¹⁸⁻²⁰, via the cholinergic axon reflex. This reflex then in turn increases acetylcholine release from cholinergic nerves, which subsequently activates endothelial cell muscarinic receptors¹⁹. Also, nicotine induced cutaneous vasodilation may be associated with the activation of nicotinic receptors located on endothelial cells²¹. If aging impairs acetylcholine-induced cutaneous vasodilation, as mentioned above^{9,10}, this could be reflected as either an attenuated muscarinic- or nicotinic-receptor mediated cutaneous vasodilatory response, or a combination of both. However, further studies are required to elucidate these possible mechanisms.

Adenosine triphosphate (ATP) is thought to be released from erythrocytes, skeletal muscle, endothelial cells, and sympathetic nerves^{22,23}. Previous work demonstrated that ATP is a

potent modulator of cutaneous vasodilation (i.e., purinergic cutaneous vasodilation) in young adults^{14,24,25}; a response presumably mediated through activation of purinergic receptors located on endothelial cells. Therefore, ATP appears to be important in modulating cutaneous vasodilation in response to physiological stimuli through its effects on cutaneous microvascular endothelial cell function. A previous study reported that aging attenuates conduit artery vasodilation in response to ATP administration²⁶. However, whether aging attenuates ATP-induced cutaneous vasodilation remains to be elucidated. Thus, in the present study we evaluated the hypothesis that aging attenuates cutaneous endothelium-dependent microvascular responses to muscarinic, nicotinic, and purinergic receptor activation in men.

MATERIALS AND METHODS

Ethical approval

This study was approved by the University of Ottawa Health Sciences and Science Research Ethics Board and conformed to the standards outlined in the *Declaration of Helsinki* with the exception that registration in a database was not done in the present study. All volunteers read and provided their signed informed consent form before their participation.

Participants

Twenty-two young (n=11) and older (n=11) males took part in the study. Since we previously observed that aging effects on cutaneous microvascular responses in males differ from those observed in females^{27,28}, we elected to exclude females to avoid any sex-related differences. The participants' physical characteristics are presented in Table 1. None of the participants reported a history of specific medical conditions that may affect cutaneous perfusion (i.e., cystic

fibrosis transmembrane conductance regulator mutations, skin disorders, hypertension, heart disease, diabetes, or autonomic disorders), and none were smokers or had smoked in the past 10 years. Also, none of the participants were on prescription medications. During the preliminary session, height was assessed using an eye-level physician stadiometer (Model 2391, Detecto Scale Company, Webb City, MO, USA), and body mass was determined using a digital weight scale platform with a weighing terminal (Model IND560, Mettler Toledo Inc., Schwerzenbach, Switzerland). Resting arterial blood pressure in a seated position was obtained by manual auscultation using a mercury column sphygmomanometer (Baumanometer Standby Model, WA Baum, Copiague, NY, USA).

Experimental session

Prior to the experimental session, all participants refrained from consuming over-the-counter medications for >48 h (e.g., nonsteroidal anti-inflammatory drugs, vitamins, and minerals), alcohol and caffeine for >24 h, strenuous physical activity for >12 h, and food for >2 h. Upon arrival to the laboratory, participants rested on a semi-recumbent bed in a thermoneutral environment (~24 °C). During this time, a 25-gauge needle was inserted into the un-anaesthetized dermal layer of the skin of the left dorsal forearm using an aseptic technique with the entry and exit points separated by ~2.5 cm. A microdialysis fibre (30 kDa cutoff, 10 mm membrane) (MD2000, Bioanalytical Systems, West Lafayette, IN, USA) was then passed through the lumen of the needle, and thereafter the needle was withdrawn. Three microdialysis fibres were placed in the skin, each site separated by ~4 cm to avoid between-site interference of drug administration. Each fibre was connected to a liquid switcher (Model 110, CMA Microdialysis AB, Kista, Sweden) to facilitate constant perfusion when changing between drug doses.

Approximately 10 min after the placement of the three microdialysis fibres, all skin sites were perfused with lactated Ringer's solution (Control, Baxter, Deerfield, IL, USA) for a period of >80 min to allow for the trauma associated with fibre insertion to subside. Perfusion at each of the skin sites was maintained at a rate of $4.0 \mu\text{l}\cdot\text{min}^{-1}$ using a micro-infusion pump (Model 4004, CMA Microdialysis, Solna, Sweden). Thereafter, a 10 min baseline measurement was initiated, after which pharmacological agents were administered at the three intradermal forearm skin sites as follows: 1) methacholine (Sigma-Aldrich, St. Louis, MO, USA) (muscarinic receptor agonist, 5 doses: 0.0125, 0.25, 5, 100, 2000 mM), 2) nicotine (MP Biomedicals, Santa Ana, CA) (nicotinic receptor agonist, 5 doses: 1.2, 3.6, 11, 33, 100 mM), or 3) ATP (Cayman Chemical, Ann Arbor, MI, USA) (purinergic receptor agonist, 5 doses: 0.03, 0.3, 3, 30, 300 mM). All doses for the respective agents were determined based on our previous work using methacholine^{14,29}, nicotine¹⁹ and ATP^{24,30}, respectively. Methacholine, nicotine, and ATP were all administered in a dose dependent manner for 25 min per dose at a rate of $4.0 \mu\text{l}\cdot\text{min}^{-1}$. After the completion of drug administration, 50 mM sodium nitroprusside (Sigma-Aldrich) was administered for 20-30 min at each microdialysis site at a rate of $6.0 \mu\text{l}\cdot\text{min}^{-1}$, which elicited maximal cutaneous blood flow. The criterion of maximal blood flow was defined as a period of a greater than 2 min where a stable plateau of cutaneous blood flow was observed.

Measurements

An integrated laser-Doppler flowmetry probe (model 413, Perimed, Stockholm, Sweden) was used for assessing cutaneous red blood cell flux, an index of cutaneous blood flow expressed in perfusion units, at a rate of 32 Hz. Each Doppler probe was connected to the laser-Doppler flowmetry recording system (PeriFlux System 5000, Perimed, Stockholm, Sweden). Manual

auscultation was performed using a mercury column sphygmomanometer (Baumanometer Standby Model, WA Baum, Copiague, NY, USA) to obtain blood pressures every 10-15 min. Cutaneous vascular conductance was evaluated by cutaneous red blood cell flux divided by mean arterial pressure (diastolic arterial pressure plus one-third the difference between systolic and diastolic pressures). Cutaneous vascular conductance was presented as %max to minimize the effect of site-to-site heterogeneity on the level of cutaneous blood flow³¹.

Data analysis

Baseline cutaneous vascular conductance was obtained by averaging values over the final 5 min of the 10-min baseline period at each of the skin sites (defined as “Baseline”). The maximum absolute cutaneous vascular conductance elicited by sodium nitroprusside administration was determined as the average response recorded over a minimum 2 min period where a stable plateau of cutaneous blood flow was observed at the three skin sites. All cutaneous vascular conductance values during the administration of methacholine, nicotine, and ATP were obtained by averaging the measurements made over the last 5 min of the 25-min infusion period at each dose. The methacholine, nicotine, and ATP concentrations required to elicit 50% of maximal cutaneous vasodilation (EC50, in mM) were evaluated with a four-parameter logistic regression as reported previously³² using commercially available software (GraphPad Prism 6.0; GraphPad Software, La Jolla, CA). In this analysis, cutaneous vascular conductance was normalized within each site by defining the smallest value as 0% and largest value as 100%. EC50 values were not identified in 2 older men since the data did not fit the logistic curve model.

Statistical analysis

Statistical analyses were conducted using SPSS 24 (IBM, Armonk, NY, USA). Based on cutaneous vascular conductance³³ obtained in our previous work with 80% power and a significance level of 0.05, a minimum sample size of 9 was determined. Cutaneous vascular conductance (%max) was analyzed with a two-way mixed-model analysis of variance with the factors of age (Young and Older) and stage (Baseline and each dose of pharmacological agent employed). When detecting a significant interaction, or a main effect, *post hoc* multiple comparisons were carried out using a modified version of the Bonferroni correction (Holm-Bonferroni method). In addition, Student's *t*-tests were employed where applicable to determine significant between-group differences including baseline (%max) and maximum absolute (perfusion units·mmHg⁻¹) cutaneous vascular conductance, EC50, and subject characteristics data (i.e., age, height, body mass, and blood pressures). The level of significance for all analyses was set at $P \leq 0.05$. All values are reported with a mean \pm 95 % confidence interval ($1.96 \times$ standard error of the mean).

RESULTS

Cutaneous vascular conductance

Maximum absolute cutaneous vascular conductance did not differ between the two groups at all skin sites (all $P > 0.05$, Table 2). Cutaneous vascular conductance increased from Baseline in response to administration of methacholine (≥ 0.0125 mM for both groups), nicotine (1.2, 11, 33, and 100 mM in young adults and ≥ 11 mM in older adults) and ATP (≥ 3 mM for both groups) (all $P \leq 0.05$, Figure 1). Cutaneous vascular conductance during methacholine (Figure 1A) or nicotine (Figure 1B) administration was similar between the two groups with the exception of an attenuated ATP-induced cutaneous vasodilation at doses of 3 and 30 mM in the older males relative

to their young counterparts (3mM:23±6 vs. 40±13%max, 30 mM:62 ± 11 vs. 83 ± 8%max, both $P \leq 0.05$, Figure 1C). Similarly, EC50 of cutaneous vasodilation was increased for ATP, but not methacholine and nicotine in older versus young males (Figure 2)

DISCUSSION

We are the first to examine whether aging modulates cutaneous vasodilation in response to muscarinic, nicotinic, and purinergic receptor specific activation in men. We showed that although muscarinic and nicotinic cutaneous vasodilation were unaffected by aging, there was an age-related reduction in purinergic receptor related cutaneous vasodilation.

Aging effect on cutaneous vasodilation

We demonstrated that ATP-induced cutaneous vasodilation was attenuated in older versus young males (Figure 1C). This was also evident by a rightward shift in the EC50 value for the older males (Figures 1C and 2C). These results suggest that aging diminishes cutaneous microvascular endothelial function associated with purinergic receptor stimulation. In line with our results evaluating cutaneous microvascular function, Mortensen et al.²⁶ demonstrated that leg conduit artery vasodilation in response to ATP was lower in older adults relative to young adults. Taken together, aging appears to attenuate the vasodilatory response to ATP, irrespective of the vascular type (i.e., conduit arteries or cutaneous microvessels).

We do not know the mechanism by which aging impairs ATP-induced cutaneous vasodilation in the present study. However, given that the study by Mortensen et al.²⁶ showed that aging lowers purinergic receptor expression in skeletal muscle, a similar reduction may occur in aged human skin, underlying the reduction in purinergic cutaneous vasodilation observed in the

older men in the current study. In addition, nitric oxide synthase may be involved in this age-related reduction in purinergic cutaneous vasodilation. This possibility is supported by the fact that nitric oxide synthase contributes to cutaneous vasodilation in response to the administration of 30 mM of ATP²⁴, and aging is associated with diminished nitric oxide synthase contribution in regulating cutaneous vasodilation³⁴⁻³⁶. Furthermore, it is possible that aging might increase ectoenzymes, which hydrolyze ATP, ultimately reducing ATP bioavailability and thereby reducing the activation of purinergic receptors. Further studies are required to elucidate this possibility.

In the present study, we observed age-related reductions in purinergic cutaneous vasodilation at moderate concentrations of ATP administration of 3 mM (Figure 1C). In parallel, we previously reported the absence of a clear nitric oxide synthase contribution to cutaneous vasodilation to 3 mM ATP²⁴. Taken together, our findings may indicate that the age-related attenuation of purinergic cutaneous vasodilation could also be associated with non-nitric oxide synthase mechanisms. We previously reported that cyclooxygenase, which can induce endothelium-dependent vasodilation through increasing prostanoids, is not involved in the regulation of ATP mediated cutaneous vasodilation²⁴. Thus, it is reasonable to conclude that this enzyme likely does not contribute to the attenuated purinergic cutaneous vasodilation observed in older adults. In addition to nitric oxide synthase and cyclooxygenase, endothelium-dependent vasodilation may be mediated by endothelium-dependent hyperpolarization, associated with potassium channel activation³⁷. No human study to date has directly evaluated the role of endothelium-dependent hyperpolarization in the regulation of ATP-induced cutaneous vasodilation. However, this mechanism may underlie the ATP-induced cutaneous vasodilation seen in young adults, as potassium channels are shown to play a role in ATP induced forearm

vasodilation³⁸. The contribution of endothelium-dependent hyperpolarization to ATP induced cutaneous vasodilation appears to be reduced in older adults, and this possibility requires further scrutiny.

We did not observe an age-related reduction in ATP-induced cutaneous vasodilation at the highest dose of ATP (i.e., 300 mM) (Figure 1C). Hence, the effect of aging on ATP-induced cutaneous vasodilation depends on the particular dose of ATP administered. We previously observed that 300 mM of ATP induced a maximum purinergic-mediated cutaneous vasodilation, as a higher dose (i.e., 600 mM) did not induce further cutaneous vasodilation²⁴. Therefore, our results indicate that aging does not impair maximal purinergic cutaneous vasodilation.

In the present study we demonstrated that nicotinic cutaneous vasodilation was similar between young and older males at all doses of nicotine (Figure 1B) as well as EC₅₀, showing that aging has no effect on this specific mediator of cutaneous vasodilation. Nicotinic cutaneous vasodilation is entirely induced via activation of muscarinic receptors at 1.2-11 mM of nicotine¹⁹, a response that occurs as a result of acetylcholine release due to a cholinergic axon reflex³⁹. Taken together, the observed lack of a between-group difference in nicotinic cutaneous vasodilation may suggest that aging does not affect cholinergic axon reflex function. We previously showed that higher doses of nicotine (33-100 mM) cause cutaneous vasodilation independently of muscarinic receptor activation¹⁹. Nicotinic receptors are found on cutaneous vascular endothelial cells²¹ and therefore their activation may mediate the vasodilatory response. In view of the fact that we observed no differences between groups in cutaneous vasodilation elicited by 33-100 mM of nicotine, it is possible to conclude that aging does not affect endothelial nicotinic receptor function.

Vascular function, specifically vascular endothelial function, is typically evaluated by acetylcholine or methacholine induced vasodilation. Our results show that methacholine-induced

cutaneous vasodilation did not differ between young and older men (Figure 1A). Consistent with this observation, previous reports have shown that acetylcholine-induced cutaneous vasodilation does not differ between young and older adults^{7,8}, albeit an age-related reduction in cutaneous vasodilation in response to acetylcholine was observed in some studies^{9,10}. However, since acetylcholine can activate both muscarinic and nicotinic receptors¹¹, the results reported in the aforementioned studies may be associated with both receptor types. Also, acetylcholine induced cutaneous vasodilation can be influenced by acetylcholinesterase, an enzyme that degrades acetylcholine. Therefore, acetylcholine induced cutaneous vasodilation can be influenced by several factors, making interpretation complicated. To the best of our knowledge, we are the first to evaluate the influence of aging on muscarinic cutaneous vasodilation using a wide concentration of methacholine, which has high resistance to acetylcholinesterase⁴⁰, suggesting that aging does not affect muscarinic vasodilation in men. In addition, it is worth noting that despite the fact that we did not observe a clear aging effect on muscarinic or nicotinic cutaneous vasodilation, we showed that aging markedly impairs purinergic cutaneous vasodilation. Taken together, our findings demonstrate that age-related effects on cutaneous vascular endothelial function are receptor specific and that aging attenuates purinergic cutaneous vascular function.

Perspectives

The present study demonstrated that aging specifically attenuates purinergic receptor related cutaneous vasodilation without affecting muscarinic and nicotinic receptor specific cutaneous vasodilation in men. Given that microvascular dysfunction may precede cardiovascular disease^{5,6}, it can be postulated that the restoration of purinergic receptor function in older men might serve as a management therapy to maintain normal or near-normal microvascular function,

thereby possibly reducing the risk of cardiovascular disease in this population group. Further research is required to investigate this possibility.

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COMPETING INTERESTS

None.

AUTHOR CONTRIBUTIONS

N.F. and G.P.K. conceived and designed experiments. N.F. contributed to data collection. N.F. performed data analysis. N.F., T.N., R.J.S., P.B., G.W.M., and G.P.K. interpreted the experimental results. N.F. drafted the manuscript. N.F., T.N., R.J.S., P.B., G.W.M., and G.P.K. edited and

revised the manuscript. All authors approved the final version of the manuscript. All experiments took place at the Human and Environmental Physiology Research Unit located at the University of Ottawa.

Table 1. Participant characteristics

	Young males	Older males
Number of subjects	11	11
Age (years)	24 ± 4	61 ± 8*
Height (m)	1.74 ± 0.06	1.76 ± 0.11
Body mass (kg)	81.2 ± 12.0	78.5 ± 14.1
Body mass index (kg/m ²)	27.0 ± 4.8	25.3 ± 3.7
Blood pressure (mm Hg)		
Systolic	119 ± 6	118 ± 10
Diastolic	79 ± 6	77 ± 7
Mean arterial pressure	93 ± 5	91 ± 7

All values are expressed as means ± standard deviation. *, indicates significant difference between young and older males ($P \leq 0.05$)

Table 2. Absolute maximal cutaneous vascular conductance at the three skin sites.

	(perfusion units mmHg ⁻¹)	
	Young males	Older males
Methacholine	2.0 ± 0.5	2.1 ± 0.5
Nicotine	1.5 ± 0.4	1.5 ± 0.4
ATP	1.8 ± 0.3	1.8 ± 0.6

Values are expressed as means ± 95% confidence interval. Data are presented in separate groups of young males (n = 11) and older males (n = 11). There were no between-site differences ($P > 0.05$ for a main effect of treatment site).

FIGURE LEGENDS

Figure 1: Cutaneous vascular response to methacholine (panel A), nicotine (panel B), and ATP (panel C) in young (open circle, n = 11) and older men (filled square, n = 11). Data are presented as mean \pm 95% confidence interval. *, indicates significant different between young and older males ($P \leq 0.05$).

Figure 2: Methacholine (panel A), nicotine (panel B), and ATP (panel C) concentrations required to induce half of the maximal cutaneous vasodilator response (EC50). The total number of participants was 11 each for both young (open bar) and older men (filled bar). However, only 9 older males were used for the nicotine data (EC50 was not identified for 2 older participants since the data did not fit the logistic curve model). Data are presented as mean \pm 95% confidence interval.

Figure 1.

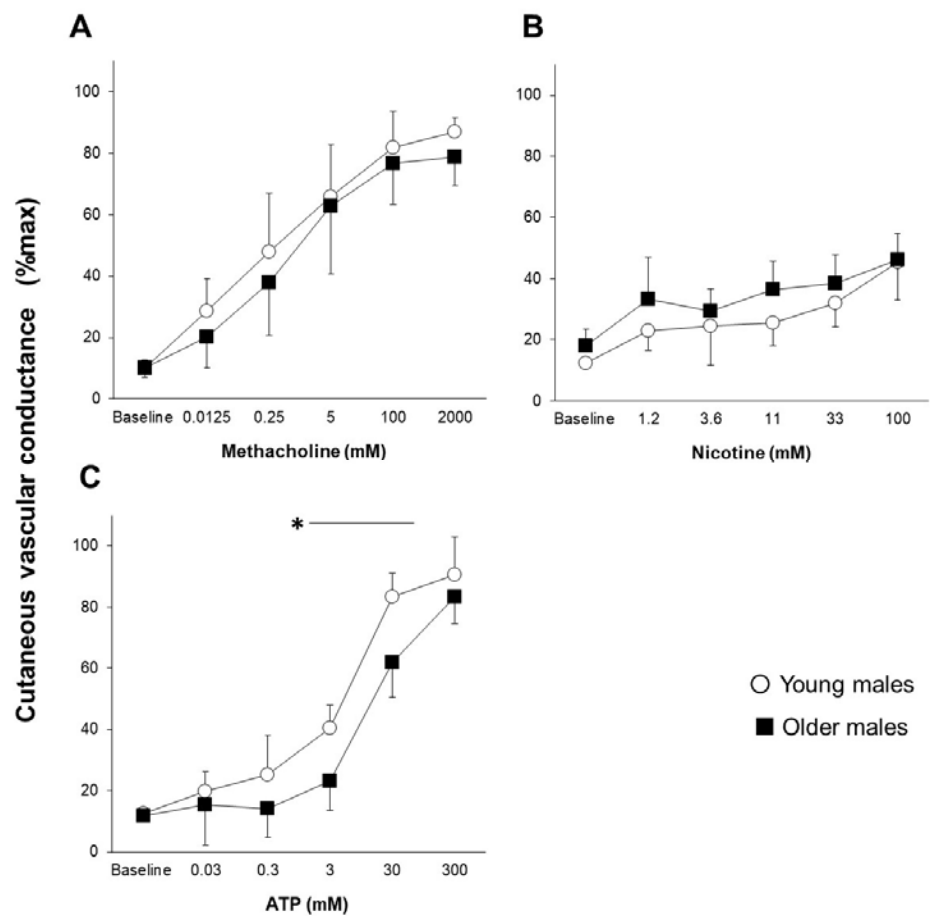


Figure 2.

