Prostacyclin, but not nitric oxide, is the major mediator of acetylcholine-induced vasodilatation in the isolated mouse heart

Paweł Gwoźdź, Łukasz Drelicharz, Valery I. Kozlovski*, Stefan Chlopicki

Department of Experimental Pharmacology, Chair of Pharmacology, Jagiellonian University Medical College, Grzegórzecka 16, PL 31-531 Kraków, Poland

*Current address: Chair of Pharmacology, Grodno Medical University, Gorky 80, 230015 Grodno, Belarus

Correspondence: Stefan Chlopicki, e-mail: s.chlopicki@cyfronet.krakow.pl

Abstract:
In many species, acetylcholine (Ach) induces coronary vasodilatation via endothelium-derived nitric oxide (NO). The aim of the present study was to examine if this rule pertains also to the coronary circulation of the mouse. We examined the involvement of NO and prostacyclin (PGI2) in the coronary flow response to Ach as compared to response to bradykinin (Bk) in hearts isolated from FVB or C57Bl/6 mice and perfused according to the Langendorff technique.

In the isolated mouse heart, response to Ach consisted of two distinct phases: immediate, transient vasodilatation/vasoconstriction (< 1 min) that differed between FVB and C57Bl/6 mice; and delayed sustained vasodilatation (up to 8 min) that was similar in FVB and C57Bl/6 mice. In FVB mice, the immediate phase of the Ach response consisted of a short-lasting vasodilatation followed by a vasoconstriction. In contrast, in C57Bl/6 mice, the immediate phase of the Ach response consisted exclusively of a short-lasting vasoconstriction. However, both in FVB and C57Bl/6 mice, the delayed vasodilatation was a major part of the coronary flow response to Ach and it was associated with an increase in 6-keto-PGF1α concentration in the effluent. L-NAME (5 × 10⁻⁴ M) displayed a minor effect on the delayed phase of the Ach response in either mouse strain. In turn, indomethacin (10⁻⁶ M), but not rofecoxib (5 × 10⁻⁶ M), completely inhibited the delayed phase of the Ach response and the concomitant PGI2 release. On the other hand, vasodilatation induced by Bk was markedly inhibited by L-NAME, while it was unaffected by indomethacin in FVB as well as in C57Bl/6 mice.

In summary, in the isolated mouse heart, Ach-induced coronary flow response displays an unusual biphasic nature and is mediated in major part by PGI2, but not by NO. Thus, in the isolated mouse heart, in parallel to Bk or other agents that are suited for the functional assessment of NO-dependent endothelial function, Ach should be used to assess PGI2-dependent endothelial function.

Key words:
acetylcholine, prostacyclin, nitric oxide, isolated mouse heart, coronary vessels, endothelium

Abbreviations:

Introduction
It is well known that acetylcholine (Ach) activates muscarinic receptors localized on the endothelium and vascular smooth muscle cells [21] and causes endothelium-dependent vasodilatation or sometimes...
vasoconstriction, depending on the vascular bed, the species studied and the functional state of the endothelium [14].

In many species, Ach-induced vasodilatation results from endothelial release of nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) or a cyclooxygenase (COX) product (PGI2). While NO is the most important endothelium-derived vasodilator released by Ach in conduit vessels, EDHF, most probably a product of cytochrome P-450-dependent arachidonate metabolism (EET) [11], plays a major role in response to Ach in resistance arteries [38].

In contrast to the overwhelming evidence on the involvement of NO and EDHF in endothelium-dependent vasodilatation induced by Ach, there is limited evidence supporting the involvement of endogenous PGI2 in this response [27].

Along with the increasing use of genetically modified mice to study cardiovascular biology, the mechanism of the vasoactive action of Ach was examined in murine aortic rings [3], isolated coronary [28, 29] or carotid conduit arteries [10] as well as in the perfused hindlimb [4]. In all these preparations Ach-induced vasodilatation was mediated by NO or EDHF. Interestingly, a role of PGI2 in Ach-induced vasodilatation was reported in the coronary resistance arteries of C57Bl/6 mice [15]. As there are significant differences in vascular biology between various mouse strains [2], it remains unknown if Ach-induced PGI2 release in coronary resistance arteries is a general phenomenon for the murine heart, or if it is limited to the C57Bl/6 mouse strain only.

The aim of the present study was, therefore, to re-examine the involvement of NO and PGI2 in Ach-induced vasodilatation as compared to bradykinin (Bk)-induced vasodilatation in hearts isolated from FVB or C57Bl/6 mice and perfused according to the Langendorff technique. In addition we assessed the contribution of muscarinic M2 and M3 receptors to Ach-induced coronary response in both strains of mice [9, 34].

Materials and Methods

Animals

All animal procedures conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the experimental procedures used in the present study were approved by the the local Ethical Committee on Animal Experiments of Jagiellonian University. Male C57Bl/6J mice and FVB mice weighting 15–25 g were used. C57Bl/6J mice were purchased from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw and bred in local animal house. FVB mice were obtained from Harvard Medical School [32] and bred in the Animal house of the Polish Academy of Science Medical Research Centre in Warsaw, and the Animal house of Jagiellonian University, Faculty of Pharmacy. The mice were maintained on 12-h dark/12-h light cycles in air-conditioned rooms with access to standard rodent diet as well as to water ad libitum.

Assessment of coronary endothelial function in the isolated mouse heart

The details of the technique of isolated heart perfusion according to Langendorff to study coronary vasodilator responses in guinea pigs or mice were described elsewhere [6, 25, 26]. Briefly, mice were anaesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg, ip). Their hearts were isolated, washed in ice-cold saline, and mounted in Langendorff apparatus by Hugo Sachs Electronics (HSE, Freiburg, Germany). Mouse hearts were perfused retrogradely through the aorta under a constant perfusion pressure of 100 mm Hg with Krebs-Henseleit buffer of the following composition (mM): NaCl 118, CaCl2 2.52, KCl 4.7, MgSO4 1.64, NaHCO3 24.88, KH2PO4 1.18, glucose 10.0, sodium pyruvate 2.0, EDTA 0.5 equilibrated with 95% O2 + 5% CO2 at 37°C in an oxygenator with rotating disc (HSE). The hearts were paced with 400 impulses per min through two platinum electrodes placed in the right atrium. Coronary flow was monitored by an ultrasonic flowmeter (HSE) and continuously displayed throughout the experiment. Coronary flow data were analyzed using specially-designed software (PSCF. EXE-IGEL, Poland).

Protocol of the experiments

Responses to Ach and Bk were assessed in the isolated heart from FVB and C57Bl/6 mice. Ach (300 pmoles) and Bk (0.1 and 1.0 nmoles) were injected as bolus injections (10 μl). In preliminary experiments Ach was given also at multiple doses (30, 100 and
300 pmoles), but in most of the cases the results for the highest dose of Ach are presented as representative. In each heart a vasodilator agent was injected twice: in the absence and in the presence of an inhibitor. In control experiments, in the absence of inhibitors, coronary vasodilator responses to both agents were reproducible (data not shown) throughout the whole experiments that lasted up to 2 h. At the end of the experiment hearts were weighed and coronary vasodilator responses were expressed in ml/min/g of wet ventricular weight.

To study the contribution of NOS and COX to the coronary flow responses, the nonselective NO-synthase inhibitor – L-NG-nitro-L-arginine methyl ester (L-NAME, 5 × 10^{-4} M) and the nonselective cyclooxygenase inhibitor – indomethacin (5 × 10^{-6} M) were used, respectively. To study the contribution of muscarinic receptors to the Ach-induced responses, the M2 and M3 receptor antagonists, methoctramine (3 × 10^{-7} M) and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, 3 × 10^{-8} M) were used, respectively. Inhibitors were infused for at least 15 min before eliciting a response.

The vasodilator agents, L-NAME, 4-DAMP and methoctramine were dissolved in saline, while indomethacin was dissolved in a 5% solution of NaHCO3. Fresh solutions of all pharmacological tools were prepared ex tempore prior to the experiments.

**Measurements of 6-keto-PGF1α in the effluent from the isolated heart**

For determination of the concentration of 6-keto-PGF1α in the effluent, samples of effluent (500 μl) were collected in Eppendorf tubes, at basal conditions and after stimulation with Ach, in the absence or the presence of COX inhibitors; indomethacin (10^{-6} M) or rofecoxib (10^{-6} M). Samples of effluent were taken before Ach response and at the peak of flow response induced by Ach. Samples were stored at −70°C until assayed using the commercially available enzyme immunoassay kits (Cayman Chemical Co, Ann Arbor, MI). Concentration of 6-keto-PGF1α in effluent was expressed in pg/ml of coronary flow.

**Statistical analysis**

Results are presented as the mean ± SEM. The difference between means was evaluated using paired Student’s t-test. A value of p < 0.05 was considered to be statistically significant.

---

**Results**

**Coronary flow response to Ach in the isolated mouse heart**

As shown in Figure 1, the coronary vasodilator response to Ach (0.3 nmoles) in the isolated mouse heart of FVB or C57Bl/6 mice included two phases: the immediate, transient response and the delayed, sustained response. The immediate phase of the Ach response differed between FVB and C57Bl/6 mice, while the delayed phase of the Ach response was similar in FVB and C57Bl/6 mice.

In FVB mice the immediate phase of the Ach coronary flow response consisted of short-lasting vasodilatation followed by vasoconstriction (Fig. 1A). In C57Bl/6 mice the initial vasodilatation was absent, so the immediate response to Ach consisted of vasoconstriction exclusively (Fig. 1B). In both FVB and C57Bl/6 mice, the delayed sustained vasodilatation was a major part of the Ach response and lasted up to
8 min in contrast to the short-lasting immediate phase of this response (lasting less than 1 min).

In FVB mice, the nonselective NOS inhibitor, L-NAME (5 × 10^{-4} M) completely inhibited the immediate transient vasodilatation induced by Ach, augmented the subsequent vasoconstriction (by 80 ± 40%), while the delayed part of the Ach response was inhibited by L-NAME only by 19 ± 6% (Fig. 2A). L-NAME also mildly inhibited the delayed vasodilatation induced by Ach in C57Bl/6 mice (by 16.99 ± 5%) (Fig. 2A). In both FVB and C57Bl/6 mice, basal coronary flow was slightly decreased by L-NAME (by 23.49 ± 8% and 25.15 ± 10%, respectively).

Neither in FVB nor in C57Bl/6 mice the nonselective COX inhibitor indomethacin (10^{-6} M) significantly affected the immediate phase of the Ach coronary flow response. However, in both FVB and C57Bl/6 mice indomethacin almost completely inhibited the delayed phase of coronary flow response to Ach (by 78.67 ± 14 and 97.37 ± 2%, respectively) (Fig. 2B). Indomethacin had no effect on basal coronary flow either in FVB or in C57Bl/6 mice.

M_2 receptor antagonist methoctramine (3 × 10^{-7} M) had no effect on Ach-induced delayed coronary vasodilation in both FVB and C57Bl/6 mice, whereas M_3 receptor antagonist 4-DAMP (3 × 10^{-8} M) completely abolished this response in both strains of mice (Fig. 3). Short-term coronary vasoconstrictor response to acetylcholine (0.3 nmoles) was also not influenced by methoctramine in both FVB mice (4.8 ± 1.0 ml/min/g before methoctramine and 4.8 ± 1.0 ml/min/g after methoctramine, n = 4) and C57Bl/6 mice (9.8 ± 2.0 ml/min/g before methoctramine and 10.4 ± 1.8 ml/min/g after methoctramine, n = 5). This phase of Ach response was however, completely abolished by 4-DAMP in both FVB mice (6.0 ± 1.0 ml/min/g before 4-DAMP and 0.2 ± 0.2 ml/min/g after 4-DAMP, n = 3) and C57Bl/6 mice (10.2 ± 2.0 ml/min/g before 4-DAMP and 0.2 ± 0.2 ml/min/g after 4-DAMP, n = 5).
In contrast to the response to Ach, L-NAME profoundly inhibited the response to Bk in FVB and C57Bl/6 mice (for 1 nmole of Bk by 51.85 ± 11% and 37.31 ± 8%, respectively) (Fig. 4). On the other hand, indomethacin had no effect on the vasodilatory response to Bk (0.1 and 1.0 nmols) both in FVB and C57Bl/6 mice (Fig. 4).

**Measurement of 6-keto-PGF₁₀ in the effluent from the isolated mouse heart**

Injection of Ach (300 pmoles) resulted in a significant increase in the concentration of 6-keto-PGF₁₀ in the effluent (Fig. 5), and this effect was abrogated by indomethacin (5 × 10⁻⁶ M) (by 96.56 ± 0.1%), while rofecoxib (5 × 10⁻⁶ M) was without an effect (382.14 ± 45.0 vs. 396.7 ± 80.0 pg/ml before and after rofecoxib, respectively).

**Discussion**

In the present work we demonstrated that in the coronary circulation of FVB and C57Bl mice, Ach-induced coronary flow response was dependent on M₃ muscarinic receptors [21, 23], displayed an unusual biphasic nature and was mediated in major part by PGI₂, but not by NO. Although in FVB mice the immediate phase of the Ach response, consisting of short-lasting vasodilation, was inhibited by L-NAME, this phase of the Ach response was virtually absent in C57Bl/6 mice. In turn, in both strains of mice, the major part of the Ach response, the delayed vasodilatation, was almost completely inhibited by indomethacin. Furthermore, Ach induced the release of PGI₂, as evidenced by an increase in 6-keto-PGF₂α concentration in the effluent. This response was also abolished by indomethacin, further confirming the major role of PGI₂ in Ach-induced vasodilatation in the isolated murine heart. Lack of the effect of the selective COX-2 inhibitor, refecoxib on Ach-induced coronary flow response and PGI₂ release suggests that endothelial PGI₂ released by Ach was derived from COX-1, but not from COX-2. These results are consistent with the current understanding of the regulation of COX-1 activity in response to endothelial activation in a physiological situation. Taking into consideration a recent finding on the involvement of COX-2 in endothelium-
dependent vasodilatation in diabetes [1], it would be interesting to test the role of COX-2 in the coronary vasodilator response to Ach in mice with a vascular pathology.

Interestingly, in both FVB and C57Bl/6 mice, delayed vasodilatation was slightly, albeit consistently reduced in the presence of L-NAME. It has been previously demonstrated that NO might have played a permissive role in PGI2-mediated vasodilatation [20]. Indeed, NO may enhance COX activity and activate the production of eicosanoids [7, 37]. However, this seems an unlikely explanation for the effect of L-NAME on Ach-induced delayed vasodilatation in coronary circulation of healthy FVB mice, since L-NAME did not influence the PGI2 release by Ach in this preparation (data not shown). Alternatively, NO could have contributed to this response by increasing the sensitivity of vascular smooth muscle to PGI2 action, by the modulation of intracellular PDE activity or by other mechanisms [17, 31].

In previous studies in various murine vascular beds [3, 10, 29], the major role of NO in Ach-induced vasodilatation was reported. In turn, in the coronary circulation of the rabbit [27] vasodilatation induced by Ach was partially mediated by PGI2, while in guinea-pig hearts entirely by NO [5, 39]. In contrast, in rats Ach coronary evoked coronary vasoconstriction, not vasodilatation [21]. Here, in agreement with previous studies, we demonstrated that PGI2 was the major mediator of response to Ach in the isolated murine heart [15, 16] and this pertains not only to C57Bl/6 mice but also to FVB mice. We characterized the biphasic pattern of the response to Ach and revealed slight differences in the pattern of the immediate phase of the Ach response between FVB and C57Bl/6 mice i.e. the presence and absence of the NO-dependent transient vasodilatation, respectively. In both strains of mice, the initial phase of the Ach response included also a transient vasoconstriction that could be linked to the direct action of Ach on vascular muscle [21] or to the endothelium-derived TxA2 [40]. In the presence of L-NAME but not indomethacin, vasoconstriction induced by Ach was potentiated, suggesting the modulatory role of NO in this phase of the Ach response.

We showed that both coronary vasodilator and vasoconstrictor responses were mediated by muscarinic M3 receptors. Our results are in line with previous work in knock-out mice for genes of muscarinic M3 and M2 receptors [30] as well as with the pharmacological results from other species [35, 36]. In contrast, it was also reported that muscarinic M2 receptors mediated Ach-induced coronary vasoconstriction [33] and Ach-induced release of prostaglandins [22]. Our results did not confirm the contribution of muscarinic M2 receptors to the effects of Ach in the coronary vasculature of FVB and C57Bl/6 mice. The reason for this discrepancy is not clear.

In contrast to the coronary flow response to Ach, in both mouse strains, the coronary flow response to BK was markedly inhibited by L-NAME but not by indomethacin. Accordingly, BK-induced vasodilatation was mediated by NO, but not by PGI2. Still the involvement of the NO-independent component of the BK response was visible in our experiments that could be attributed to EDHF, most likely to EETs [8, 11].

To summarize, we showed that in murine coronary circulation, the vascular response to Ach displayed an unusual biphasic pattern and was mediated in major part by endothelial COX-1-derived PGI2. In contrast, NO was a major mediator of the BK response.

Although PGI2 and NO seem to be released from the endothelium in a coupled manner [19], the endothelial dysfunction that occurs in various cardiovascular pathologies and is characterized by an impaired production of NO, may be linked to the impairment of basal PGI2 production [13, 18, 24], the preservation of PGI2 production or even the compensatory increase in the production of PGI2 [12]. Apparently, in the isolated mouse heart, in parallel to BK or other agents that are used for the functional assessment of NOS-dependent endothelial function, Ach may be used to assess COX-1/PGI2-dependent vascular function. This approach may prove efficient to track simultaneously functional alterations in NO and PGI2 pathways in coronary circulation of genetically-modified mice with various cardiovascular pathologies and endothelial dysfunction.

Acknowledgment:
This work was supported by the Polish Ministry of Science and Higher Education (grant no. PBZ-KBN-101/709/2003), and by NATO Collaborative Linkage Grant (CGL 982/76). Professor Stefan Chlopicki is the recipient of a Professorial Grant from the Foundation for Polish Science (SP/04/04). The authors would like to thank Anna Kowalczyk (Medical Research Centre, Warszawa) and Anna Obrusnik (Jagiellonian University, Krakow) for the breeding and care of the animals.

References:


Received: June 19, 2007; in revised form: September 23, 2007.