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Systemic Blood Pressure Response to the Inhibition of Two Hyperpolarizing Pathways: a Comparison to NO-synthase Inhibition

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Summary

The impact on blood pressure of two vasodilating mechanisms, underlied by vascular smooth muscle hyperpolarization, was studied and compared to that induced by nitric oxide (NO) mechanism. Systemic blood pressure, after inhibitory intervention in arachidonic acid metabolism (cytochrome P-450 inhibition by miconazole 0.5 mg/100 g b.w.), one of the hyperpolarizing pathways, did not change. After the inhibition of the action voltage-dependent K^+ channels operator (by 4-aminopyridine 0.1 mg/100 g b.w.), the other hyperpolarizing pathway, blood pressure declined slightly (from 132.3±3.2 mm Hg to 116.5±5.0 mm Hg, $P<0.05$). Inhibition of nitric oxide production (L-NAME 5 mg/100 g b.w.) increased blood pressure considerably (123.5±2.7 mm Hg to 155.4±3.1 mm Hg, $P<0.001$). After inhibition of the hyperpolarizing pathway by miconazole, hypotension induced by acetylcholine (Ach, 10 μg) represented 63.0±1.9 mm Hg vs control value 78.6±5.2 mm Hg ($P<0.001$), by bradykinin (BK) (100 μg) 59.4±3.9 mm Hg vs control value 71.2±6.1 mm Hg ($P<0.05$). After inhibition of the hyperpolarizing pathway by 4-aminopyridine, hypotension induced by ACh (10 μg) achieved 64.6±2.5 mm Hg vs control value 78.4±2.8 mm Hg ($P<0.001$) and that induced by BK (100 μg) 56.6±5.3 mm Hg vs control value 72.3±2.5 mm Hg ($P<0.001$). ACh or BK hypotension after the inhibition of the above hyperpolarizing pathways was significantly attenuated. On the contrary, after NO-synthase inhibition the hypotension to ACh was significantly enhanced. Blood pressure decrease after ACh (10 μg) hypotension was 91.8±4.1 mm Hg vs control value 79.3±3.3 mm Hg ($P<0.01$), and after BK (100 μg) it was 78.4±7.1 mm Hg vs control value 68.3±5.2 mm Hg. A different basal BP response, but equally attenuated hypotension to Ach and BK, was detected after the inhibition of two selected hyperpolarizing pathways. In cotrast, the inhibition of NO production elicited an increase in systemic BP and augmentation of ACh and BK hypotension. The effectiveness of further hyperpolarizing mechanisms in relation to systemic BP regulation and nitric oxide level remains open.

Key words

Hyperpolarizing factors • Nitric oxide • Systemic blood pressure • Hypertension • Acetylcholine • Bradykinin

Introduction

Vivid metabolism has been revealed in endothelial cells, and ensuing metabolites were shown to have a vasoactive effect. In particular, previous 25 years a large number of studies were devoted to nitric oxide, a

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"waste" product (Nathan 1992) of the catalysis of arginine to citrulline, running under NO-synthase (NOS) in endothelial cells. Nitric oxide was supposed to penetrate by simple diffusion to smooth muscle cells in tunica media, to activate guanylate cyclase and to release them *via* cGMP. This idea was later modified due to new findings. Indeed, catalysis of arginine to citrulline also takes place in vascular smooth muscle cell itself, since NO-synthase was detected in vascular smooth muscle cells (Segal *et al.* 1999, Buchwalow *et al.* 2004). NO levels measured directly *in vivo* in anesthetized dogs, close to endothelial cells of the femoral artery, were decreased only about 13 % after NOS inhibition (Gerová *et al.* 1998). Furthermore, acetylcholine, bradykinin and substance P, used as activators of NO-synthase, induce a relaxation of vessel segments *in vitro,* even after the inhibition of NO-synthase. Hyperpolarization of smooth muscle cell membrane in the media was demonstrated to underlie this relaxation. Consequently, a search for an enigmatic endothelium-derived hyperpolarizing factor (EDHF) started (Bolton and Clapp 1986, Feletou and Vanhoutte 1988, Harder *et al.* 1994, Hecker *et al.* 1994, Bakker and Sipkema 1997, Jackson 2000, Török 2000, Oltman *et al.* 2001).

Our own studies of the NO role in the integrated response of vascular tree to ACh and BK, activators of NO-synthase, also provided surprising findings. In anesthetized rats, Ach- and BK-induced hypotension was even amplified after short-lasting inhibition of NO-synthase (Gerová 1999). Persistent Ach-induced hypotension after NO-synthase inhibition was found by Mügge *et al.* (1991) in the rabbit hindlimb vascular area, and by Zanchi *et al.* (1995) and Kakizoe *et al.* (1998) who followed systemic blood pressure changes. Moreover, the enhanced hypotension to ACh and BK was also present in animals in which the inhibition of NO-synthase lasted for 6 weeks and induced a stabilized hypertension (Gerová 1999).

The investigation of K^+ channels opening in smooth muscle of conduit arteries was carried out in several directions: NO itself was suggested as hyperpolarizing factor, besides its activation of guanylate cyclase (Bolton and Clapp 1986, Bolotina *et al.* 1994, Cohen *et al.* 1997). Convincing evidence involving arachidonic acid, and its metabolite epoxyeicosatrienoic acid, in hyperpolarization of smooth muscle cells was provided by Hecker *et al.* (1994), and Campbell *et al.* (1996) who identified this metabolite chemically. Potassium efflux from endothelial cells was supposed to reach the membrane of vascular smooth muscle (VSM) and trigger its hyperpolarization (Edwards *et al.* 1998). The role of gap junctions in ionic transfer between endothelial cells and smooth muscle cells in hyperpolarization was considered by Chaytor *et al.* (2001). This idea was based on the presence of heterogenous gap junctions found just in the large conduit arteries that often served as the experimental object in studies of EDHF (Kristek and Gerová 1992, 1997, Bény and Paccica 1994). Reactive oxidative species were also considered in the search for the putative candidate of EDHF (Bény and von der Weid 1991, Matoba *et al.* 2000). Recently, an endothelium-derived natriuretic peptide was added on the list of EDHF candidates (Ahluwalia and Hobbs 2005). According to the ample body of data, a consent has emerged that a multitude of EDHFs might underlie hyperpolarizing relaxation of VSM induced by acetylcholine, bradykinin or other stimulators.

Whereas a majority of studies was performed on conduit arteries *in vitro*, a few *in vivo* studies investigated vascular areas, representing small resistant vessels: rat hindquarter perfusion, renal vascular bed, coronary microcirculation, even the human forearm area (Widmann *et al.* 1998, Fulton *et al.* 1998, Nishikawa *et al.* 1999, Harada *et al.* 2000, Halcox *et al.* 2001, Berg 2002, Pomposiello *et al.* 2003). General consensus expressed in several studies suggests that while nitric oxide operates prevalently in large arteries, the EDHF(s) operate predominantly in small resistant arteries (Nagao *et al.* 1992, Harder *et al.* 1994, Nishikawa *et al.* 1999, Oltman *et al.* 2001).

In our study we raise the question of how the impairment of hyperpolarization of vascular smooth muscle in individual vascular segments and areas is integrated, and reflected in the systemic blood pressure. In particular, the aim was to search whether EDHF participates in hypotension elicited by acetylcholine and bradykinin which persists after the inhibition of NO synthase.

From various suggested EDHF candidates we chose the following two: 1) a metabolite of arachidonic acid – epoxyeicosatrienoic acid (EET), studied in many experiments on isolated vessels including the human forearm (for review see Fleming 2001, Busse *et al.* 2002), and 2) a suggested operator of voltage-dependent K^+ channel (K^+v) which was studied in several experiments on isolated vessels (Petersson *et al.* 1997, Nishikawa *et al.* 1999). Berg (2002) provided convincing evidence on

the involvement of K^+v channels in the *in vivo* regulation of the tone of resistance vessels.

To analyze EDHF effects, the inhibitors of the two above hyperpolarizing pathways were used: miconazole and 4-aminopyridine, respectively (Petersson *et al.* 1997, Nishikawa *et al.* 1999, Halcox *et al.* 2001, Berg 2002). Two aspects were studied after the inhibition of the respective hyperpolarizing pathways: 1) the effect on basal tone of vascular smooth muscle which is reflected by systemic blood pressure, and 2) the hypotensive response of blood pressure to acetylcholine and bradykinin. Both parameters were compared to those found after the inhibition of NO-synthase with consequent lowering of nitric oxide level.

Methods

Wistar male rats (10-12 weeks of age, body weight 230-300 g) were used for the experiments. The method and experimental protocol was approved by the Animal Care and Use Committee of the Slovak Academy of Sciences. Three groups of animals were used:

Group 1 - eight animals, with inhibition of the enzyme cytochrome P-450, inducing an attenuation of metabolism of arachidonic acid to epoxyeicosatrienoic acid, supposed to be the EDHF (Campbell *et al.* 1996). The respective inhibitor miconazole (Sigma, 0.5 mg/100 g b.w., diluted in 0.5 ml salt solution) was administered intravenously during 90 s.

Group 2 - ten animals, with inhibition of the action of voltage-dependent K^+ channel operator by 4-aminopyridine (4-AP, Sigma, 0.1 mg/100 g b.w., diluted in 0.3 ml salt solution) which was administered intravenously for a period of 90 s.

Group 3 - eight animals, with inhibition of NO-synthase by N^G-nitro-L-arginine-methyl ester (L-NAME, Sigma, 5 mg/100 g b.w., diluted in 0.3 ml salt solution). L-NAME which was administered intravenously during 90 s.

Experimental protocol

The animals were anesthetized by sodium pentobarbital in the dose of 50 mg/kg, intraperitoneally. The right jugular vein was prepared and cannulated for drug administration. Heparin 25 IU was administered immediately. The right carotid artery was prepared, cannulated and connected to a Statham pressure transducer. Blood pressure was recorded on Physioscript Schwarzer.

In each group, after preparation and cannulation the vessels, a time period of 10-15 min was necessary for stabilization of blood pressure. Thereafter acetylcholine in the dose 1 μg and 10 μg, and bradykinin 100 μg, each dose diluted in 0.1 ml physiological salt solution, were administered intravenously during a constant 10 s period. The drugs were administered in a random order. The time period between administration of individual drugs was 10 min. Then the appropriate inhibitor was administered intravenously, during a 90 s time period. After blood pressure was stabilized, the administrations of acetylcholine and bradykinin were repeated in the same way as in the first series, before the inhibition of the respective hyperpolarizing pathway (again in a random order).

Statistics

The obtained experimental data were expressed as means ± S.E.M. Analysis of variance and Student´s t-test were used, for statistical analysis. Values were considered significant at *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

Group 1 Animals with the inhibition of cytochrome P-450 by miconazole

The steady-state value of systemic blood pressure was 132.7±5.9 mm Hg. After administration of miconazole, an inhibitor of cytochrome P-450, the blood pressure stabilized at 125.8±4.4 mm Hg; but this blood pressure decrease was not significant (Fig. 1).

The hypotensive response to acetylcholine in the dose of 1 μg and 10 μg represented 54.3±6.0 mm Hg and 78.6±5.2 mm Hg, respectively. After administration of miconazole, the hypotension achieved 46.0±2.5 mm Hg and 63.0±1.9 mm Hg ($P<0.001$), respectively, values being significantly smaller than those before miconazole administration (Fig. 2)

The hypotensive response to BK 100 μg before miconazole was 71.2±6.1 mm Hg and it was significantly attenuated 59.4±3.9 mm Hg ($P<0.05$) after administration of miconazole (Fig. 2).

Group 2 Animals with the inhibition of the action of K^+v channel operator by 4-aminopyridine (AP)

Systemic blood pressure in the steady-state situation was 132.3±3.2 mm Hg. After 4-AP administration BP fluctuated at lower values, reaching 116.5±5.0 mm Hg ($P<0.05$) in 2 hours, and 135.1±3.9 mm Hg in 6 hours (Fig. 1).

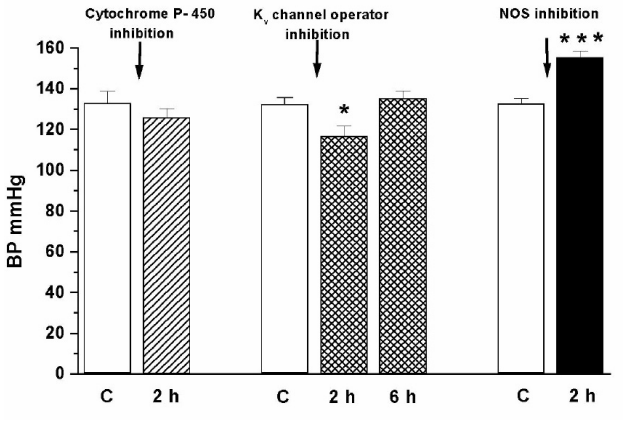

Fig 1. Systemic blood pressure before (white columns) and after inhibition of cytochrome P-450 (hatched column), K^+v activator inhibition (cross-hatched columns) and NO-synthase inhibition (black column). $^*p<0.05$, $^{***}p<0.001$

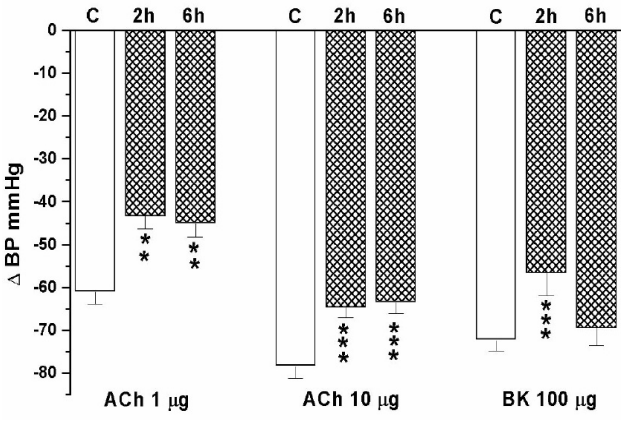

Fig. 3. Blood pressure response to ACh and BK before (white columns) and after inhibition of the action of K^+v operator by 4-AP (cross-hatched columns). $^{**}p<0.01$, $^{***}p<0.001$

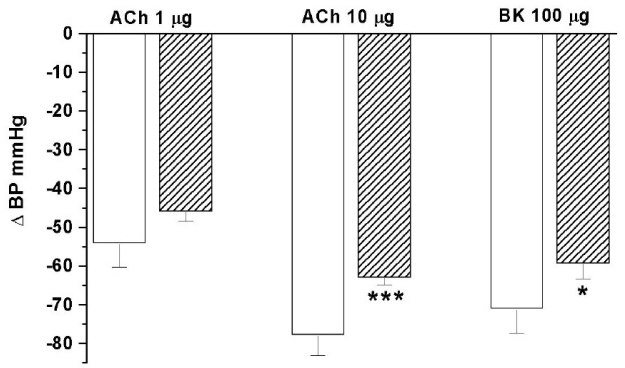

Fig. 2. Blood pressure response to ACh and BK before (white columns) and after inhibition of cytochrome P-450 inhibition by miconazole (hatched columns). $^*p<0.05$, $^{***}p<0.001$

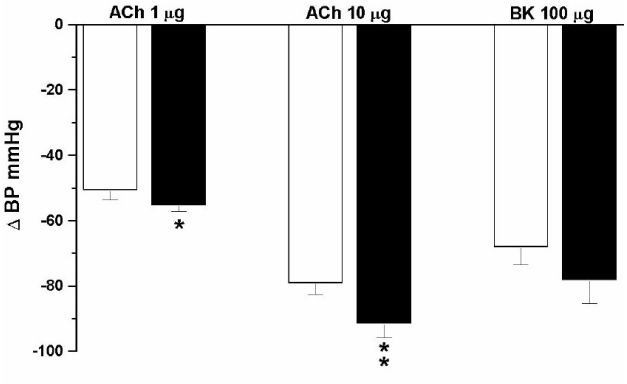

Fig. 4. Blood pressure response to ACh and BK before (white columns) and after inhibition of NO-synthase by L-NAME (black columns). $^*p<0.05$, $^{***}p<0.001$

The hypotension induced by ACh 1 μg and 10 μg was 61.0±2.9 mm Hg and 78.4±2.8 mm Hg, respectively. Two hours after administration, 4-AP, hypotension was 43.3±3.0 mm Hg ($P<0.01$) and 64.6±2.5 mm Hg ($P<0.001$) respectively. Six hours after 4-AP administration the hypotension was 45.0±3.2 mm Hg ($P<0.01$) and 63.4±2.7 mm Hg ($P<0.001$), respectively (Fig. 3).

The control values of hypotensive response to BK was 72.3±2.5 mm Hg. After 4-AP administration the hypotension was significantly attenuated to 56.6±5.3 mm Hg ($P<0.001$) in 2 hours, and it was 69.5±4.1 mm Hg in 6 hours (Fig. 3).

Group 3 Animals with NO-synthase inhibition by administration of L-NAME.

Systemic BP (132.5±2.7 mm Hg) increased to 155.4±3.1 mm Hg ($P<0.001$) in 2 hours after L-NAME administration (Fig. 1).

ACh administered in the doses 1 μg and 10 μg elicited hypotension 50.9±4.7 mm Hg and 79.3±3.3 mm Hg respectively. After L-NAME administration, the response was significantly enhanced from 53.5±3.8 mm Hg ($P<0.05$) to 91.8±4.1 mm Hg ($P<0.01$).

Bradykinin (100 μg) induced a BP decrease by 68.3±5.2 mm Hg, and after L-NAME administration the response was 78.4±7.1 mm Hg (Fig. 4); the value which tended to increase, but not significantly.

Discussion

The aim of the present study was to provide lacking data on the integrated response of systemic BP affected by the impairment of the hyperpolarization of vascular smooth muscles. Out of the multitude of possible existing hyperpolarizing pathways, two were

selected for our investigation. The experiments demonstrated in anesthetized rats that the inhibition of two hyperpolarizing pathways: one exerted by the metabolite of arachidonic acid, epoxyeicosatrienoic acid, the other by endogenous operator of K^+v channels, affected the basal systemic blood pressure differently.

Intervention in the arachidonic acid metabolism *via* inhibition of cytochrome P-450 by miconazole, did not affect the basal systemic blood pressure, while 4-AP, antagonist of K^+v channels, surprisingly slightly lowered systemic blood pressure. The results do not support the analogous role of the two EDHF pathways studied in the regulation of vascular smooth muscle tone in normotensive animals. In particular, the latter finding was unexpected and surprising.

Considering these findings in relation to the effect of inhibition of nitric oxide production and consequent blood pressure increase, the differences are even more striking. Bauersachs *et al.* (1996) considered that there is a negative feedback relation between EDHF, in particular epoxyeicosatrienoic acid, a metabolite of arachidonic acid, and nitric oxide. Even if one accepts the plausibility of this idea, it is necessary to take into consideration further aspects, at least quantitative relations in the balance between nitric oxide and the respective EDHF. The experiments indicated that lowering the epoxyeicosatrienoic acid, one of the supposed EDHFs, by miconazole, might be compensated by nitric oxide; basal blood pressure does not change. However, lowering nitric oxide after NO-synthase inhibition, was not substituted by the mentioned EDHF and resulted in an increase in basal blood pressure. One can speculate that at least the vasodilating efficacy of epoxyeicosatrienoic acid and nitric oxide are substantially different.

Thus, our experiments demonstrated that the efficiency of the two hyperpolarizing pathways seems to be quantitatively different and this fact is reflected by different changes of the basal blood pressure. After the respective inhibitory interventions the former one did not change, the latter slightly decreased the basal blood pressure. On the contrary, lowering the nitric oxide level by NO-synthase inhibition, basal blood pressure increased markedly, indicating a powerful effect of nitric oxide on basal blood pressure. Neither of the two hyperpolarizing pathways could compensate the compromised nitric oxide production. The results justify the search for the role of particular K^+ channels in relation to basal BP.

As far as the blood pressure response to ACh and BK after inhibition of two examined hyperpolarizing pathways is concerned, the attenuation of hypotension was found in both cases, in comparison to the values of hypotension seen in the control conditions. The finding is completely controversial to the findings in animals with NO-synthase inhibited for 2 hours and even in the case when inhibition lasted 6 weeks. Hypotension induced by ACh or BK in those animals was distinctly amplified (Gerová 1999).

The results indicate that ACh and BK are potent activators of both pathways of EDHFs and their inhibition attenuated the respective hypotension in comparison to the controls; contrary to NO-synthase inhibition when hypotension was maintained, or even amplified (Mügge *et al.* 1991, Zanchi *et al.* 1995, Kakizoe *et al.* 1998, Gerová 1999). It seems that the decreased NO production might be fully substituted by the hyperpolarizing response to acetylcholine and bradykinin.

It remains an open question to investigate the effectiveness of further vascular smooth muscle hyperpolarizing mechanisms in relation to systemic blood pressure, namely with respect also to nitric oxide levels. Moreover, the agonists or antagonists of K^+ channels might also affect other control systems of vascular smooth muscle tone, for example the whole cascade of the nervous control of cardiovascular apparatus which should be taken into account (Yen *et al.* 1985). Nevertheless, our experiments also provided evidence on the involvement of NO in the control of vascular smooth muscle tone *via* nervous control system (Gerová *et al.* 1995, 2004).

It would be useful to study the two potassium channel pathways in experimental models of hypertension. More pronounced changes in systemic BP, as well as in ACh and BK hypotensive responses might be expected. In spite of the abundant data on hyperpolarizing pathways in individual vascular segments and areas, this topic started to be taken into consideration on the pathogenetic processes only recently (Cox *et al.* 2001, Bratz *et al.* 2002, 2005).

In conclusion, the experiments demonstrated that basal systemic blood pressure is not affected by inhibition of arachidonic acid metabolism and lower levels of epoxyeicosatrienoic acid, one hyperpolarizing pathway. Blood pressure slightly declines after the inhibition of the action of K^+v channel operator, the other hyperpolarizing pathway. Hypotension induced by ACch or BK is,

however, significantly attenuated by inhibition of both vascular smooth muscle hyperpolarizing pathways. The responses differ completely from blood pressure response to lowered NO level after NO-synthase inhibition: implying an increase in basal blood pressure and an enhanced hypotension to ACh and BK.

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