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Acetylcholine and Bradykinin Induce Paradoxically Amplified Hypotensive Response in Hypertensive NO-Deficient Rats

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Summary

The hypotensive response to acetylcholine and bradykinin was studied in rats with NO synthase activity inhibited for a short period of 2 h or a long period of 6 weeks. N^G-nitro-L-arginine-methyl ester (L-NAME) was used as NO synthase inhibitor (given in a dose of 50 mg/kg either into the jugular vein, or daily in drinking water). Blood pressure was measured in the right carotid artery by a Statham pressure transducer in acute experiments, and on the tail artery by the plethysmographic method weekly in chronic experiments. During both the short- and long-lasting NO synthase inhibition blood pressure rose significantly. The heart rate decreased significantly in rats treated with L-NAME for 6 weeks. Surprisingly, the hypotensive responses to acetylcholine and bradykinin were present in both experimental groups. Paradoxically, the hypotensive responses to all three doses of acetylcholine were remarkably enhanced in rats with NO synthase inhibition lasting 6 weeks, in comparison to both age-matched controls and to rats subjected to short-lasting NO synthase inhibition. The blockade of muscarinic receptors by atropine abolished the hypotensive response to acetylcholine but not to bradykinin. The hypothetical mechanisms underlying this unexpected paradoxical phenomenon of cardiovascular control are discussed.

Key words

NO synthase • Long-term NO synthase inhibition • Acetylcholine hypotension • Bradykinin hypotension • L-NAME

Introduction

Acetylcholine has been recognized as an activator of NO synthase – an enzyme catalyzing arginine to citrulline, and generating the by-product nitric oxide (NO), which in turn relaxes vascular smooth muscle cells. Inhibition of NO synthase activity attenuated the above process with consequent inhibition of NO generation. The inhibitors of NO synthase, in principle arginine analogues, realize their action *via* competitive binding to NO synthase. (Ignarro *et al.* 1987, Palmer *et al.* 1987, Rees *et al.* 1990).

The isolated vessel *in vitro* behaves according to the reductionistic idea: depending on the dose of the inhibitor used, the relaxation of the vessel is attenuated, or inhibited completely (Rees *et al.* 1990).

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In acute experiments, Gardiner *et al.* (1990) reported that anesthetized rats responded to acetylcholine by a decrease in blood pressure after inhibition of NO synthase by N^G-nitro-L-arginine-methyl ester. Furthermore, when measuring the NO levels in the periendothelial area of the femoral artery of dogs directly using the porphyrinic biosensor, we found a significant, though attenuated increase of NO levels induced by acetylcholine after NO synthase inhibition. Accordingly, we suggested that other pathways of NO generation must probably exist in the living organism (Gerová *et al.* 1998).

On the other hand, there is a body of evidence from various laboratories, including ours, that long-term inhibition of NO synthase induces hypertension (Ribeiro *et al.* 1992, Morton *et al.* 1993, Arnal *et al.* 1993, Bernátová *et al.* 1996, Gerová *et al.* 1996, Sládek *et al.* 1996). A failure of nitric oxide generation with consequent failure of relaxation of vascular smooth muscles is supposed to underlie this pathological process.

The above facts appear to display certain contradictions: (i) A relatively high level of nitric oxide is always maintained after NO synthase inhibition, and acetylcholine further triggers its significant increase, (ii) long-term inhibition of NO synthase increases blood pressure due to insufficient vascular smooth muscle relaxation, induced by the lack of nitric oxide.

The first question which we posed was whether it is possible that it is only the long-term inhibition of NO synthase that compromises the catalysis of arginine to citrulline and the generation of nitric oxide? The only study addressing this question offered a negative response (Zanchi *et al.* 1995).

The next question was associated with the previous consideration: (i) Does the persisting hypotensive response after NO synthase inhibition concern only acetylcholine as a triggering factor, and/or muscarinic receptors activated by acetylcholine, or (ii) does the hypotensive response persist even after a completely different NO synthase activator?

With the aim of providing answers to the above questions, we followed the response of the cardiovascular system to acetylcholine, expressed by BP changes, in anesthetized rats (i) of a control group, (ii) in animals two hours after NO synthase inhibition, and (iii) in animals subjected to NO synthase inhibition for 6 weeks. The involvement of muscarinic receptors was also studied. In addition, the response to a completely different activator, bradykinin, was investigated.

Methods

The experiments were performed on male Wistar rats. The procedures and protocols used in this study were approved by the Animal Care and Use of Experimental Animals Committee of the Institute. Two groups of animals, 10 weeks old at the beginning of the experiment, were used. One group consisted of 8 animals which served as controls and then were studied 2 h after administration of the inhibitor of NO synthase L-NAME. The second group consisted of 10 animals which were administered L-NAME in the dose of 50 mg/kg/day in drinking water for a period of six weeks. Both groups were kept under the same conditions. Blood pressure was measured weekly on the tail artery by the noninvasive plethysmographic method.

On the day of the acute experiment, the animals were anesthetized by sodium pentobarbital in the dose of 50 mg/kg intraperitoneally. The right jugular vein was cannulated for the application of drugs. Immediately after cannulation, heparin was injected in a dose of 25 IU. The right carotid artery was prepared, cannulated and connected to a Statham pressure transducer. Blood pressure was recorded on Physioscript Schwarzer. After stabilization of the blood pressure, responses to acetylcholine and bradykinin were monitored.

Experimental protocol in control group and after two-hour NO synthase inhibition

As indicated above, the control animals were age-matched with those administered the inhibitor of NO synthase for six weeks. Three doses of acetylcholine were used: 1 μg, 5 μg and 10 μg. Each dose was diluted in the same volume of physiological Krebs solution, i.e. 0.1 ml, and was injected within 10 s. The intervals between individual applications were about 10 min.

Bradykinin was used as another activator of NO synthase. The preliminary experiments showed that the response to 10 μg acetylcholine was the same as the response to 100 μg bradykinin. The dose was dissolved again in 0.1 ml Krebs solution and applied within 10 s.

After the responses to three doses of acetylcholine and to bradykinin given randomly had been recorded, L-NAME (50 mg/kg) i.v. was injected for inhibition of NO synthase. During the following 30 min BP became stabilized at a higher level. About two hours after L-NAME administration, the above three doses of acetylcholine and bradykinin were given by the same procedure as before NO synthase inhibition.

Experimental protocol in animals with long-term inhibition of NO synthase

As mentioned above, after 6-week administration of L-NAME (50mg/kg/day in the drinking water), the animals were anesthetized similarly as the control animals. The right jugular vein was prepared for drug application and the right carotid artery for BP recording. Heparin, acetylcholine, bradykinin were applied in the same doses, same amount of Krebs solution, and duration of application as in the control experiments.

Finally, to block the muscarinic receptors, atropine (0.5 mg/kg) was injected i.v. After 15 min the responses to acetylcholine (10 μg) and bradykinin (100 μg) were recorded.

The data are expressed as mean ± S.E.M. Analysis of variance, Bonferroni and Student's t-test for paired and unpaired variables were used. Values were considered significant at *P<0.05, **P<0.01, ***P<0.001.

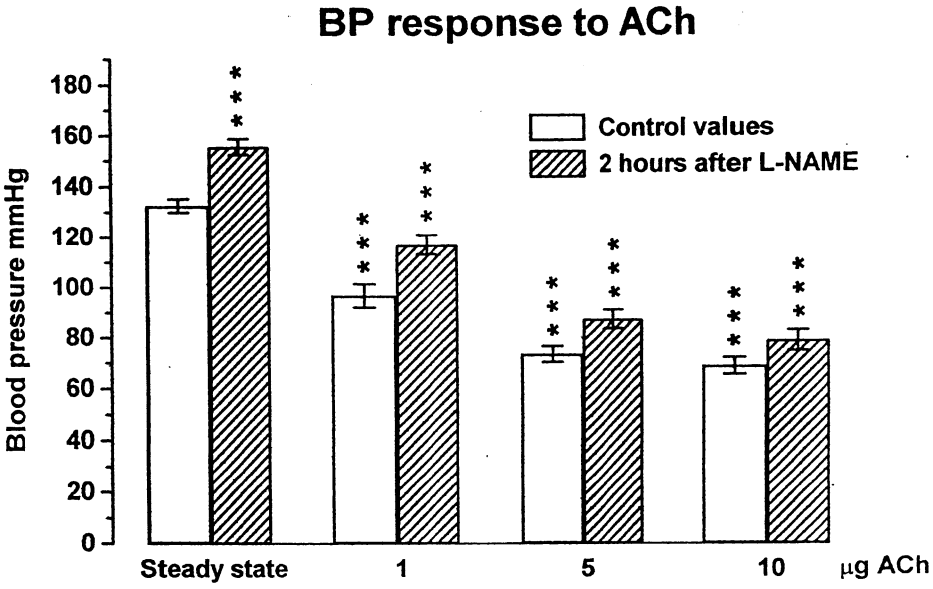

Fig. 1. *Blood pressure response (mm Hg) to acetylcholine 1 μg, 5 μg and 10 μg/0.1 ml Krebs solution, administered in 10 s. Open columns - responses in control animals, hatched columns - responses of the same animals two hours after the NO synthase inhibitor L-NAME was given. Significance: *** for p<0.001 mm Hg response to acetylcholine vs respective steady state.*

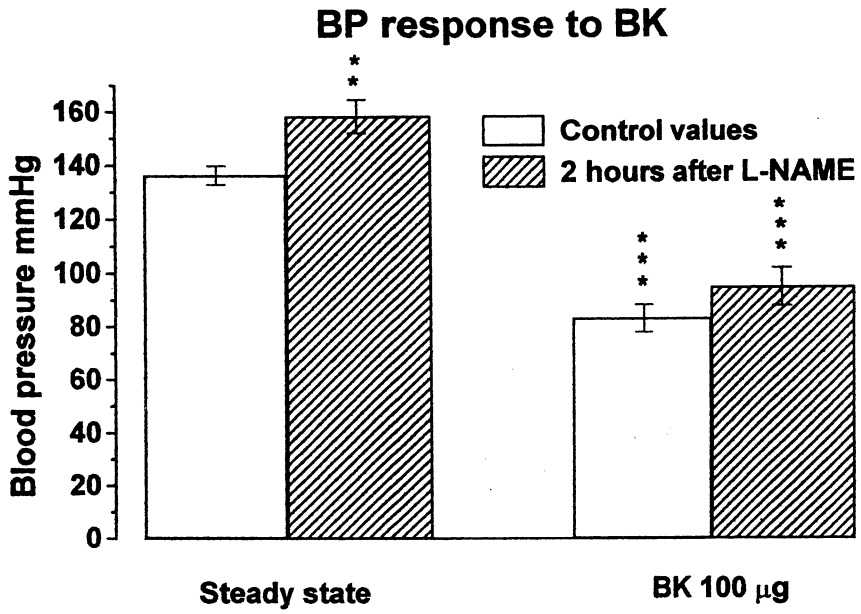

Fig. 2. *Blood pressure response (mm Hg) to bradykinin 100 μg/0.1 ml Krebs solution, administered in 10 s. Open columns - responses in control animals, hatched columns - responses of the same animals two hours after the NO synthase inhibitor L-NAME was given. Significance: response to respective steady state, ** for p<0.01 and *** for p<0.001.*

Results

Control responses and responses after two-hour NO synthase inhibition

In control animals, acetylcholine in the doses 1 μg, 5 μg and 10 μg induced a decrease of blood pressure from 132.5±2.7 mm Hg to 96.7±4.7 mm Hg (P<0.001), 73.3±3.1 mm Hg (P<0.001) and 68.7±3.2 mm Hg (P<0.001), respectively (Fig. 1). About two hours after L-NAME administration (50 mg/kg), systemic blood pressure increased from 132.5±2.7 mm Hg to become stabilized at 155.4±3.1 mm Hg (P<0.001). Acetylcholine (1 μg, 5 μg and 10 μg), applied at this blood pressure level, induced a decrease to 116.9±3.78 mm Hg (P<0.001), 87.1±3.1 mm Hg (P<0.001) and 78.6±4.1 mm Hg (P<0.001), respectively (Fig. 1).

After 100 μg bradykinin the mean blood pressure value dropped to 79.3±5.2 mm Hg (P<0.001) in control experiments. Two hours after inhibition of NO synthase, in response to the same dose of bradykinin, blood pressure dropped to 92.0±7.1 mm Hg (P<0.001) (Fig. 2).

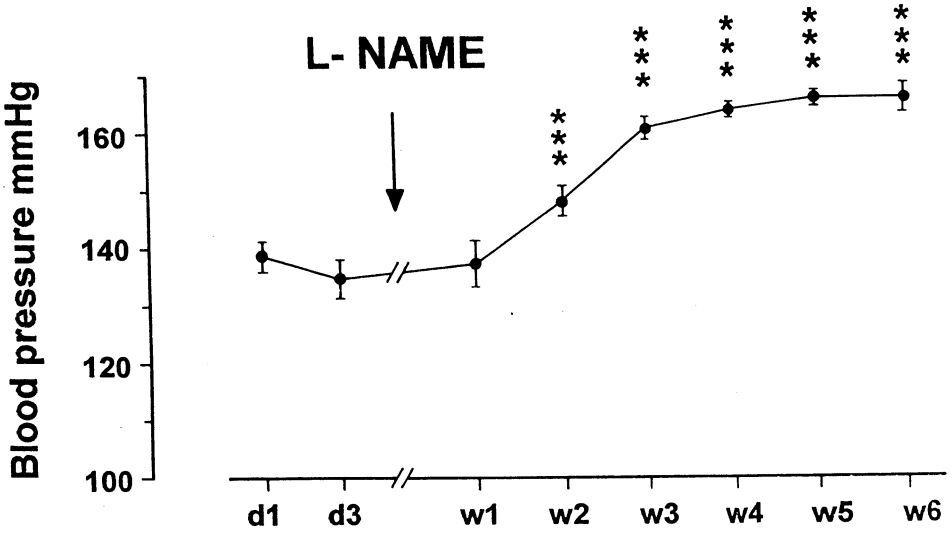

Fig. 3. *Blood pressure monitored weekly in rats administered NO synthase inhibitor L-NAME (50 mg/kg b.w. daily) in drinking water for a period of 6 weeks. Significance *** for p<0.001.*

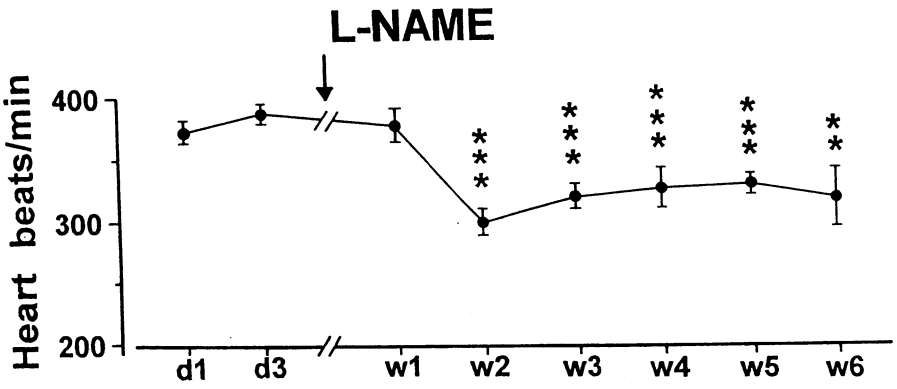

Fig. 4. *Heart rate monitored weekly in rats administered NO synthase inhibitor L-NAME (50 mg/kg b.w. daily) in drinking water for a period of 6 weeks. Significance ** for p<0.01 and *** for p<0.00.1*

Responses after 6-week NO synthase inhibition

NO synthase inhibition by L-NAME induced an increase of systemic blood pressure which was significant in the second week (from 134.5±2.5 mm Hg to 147.7±2.4 mm Hg, P<0.001). The increased blood pressure value was sustained for a period of six weeks, as shown in Figure 3, representing weekly recordings of blood pressure. In the final sixth week, the blood pressure was

Figure 3, representing weekly recordings of blood pressure. In the final sixth week, the blood pressure was 166.1±2.7 mm Hg (P<0.001). The heart rate decreased after NO synthase inhibition from 387.2±8.7 beats/min to 298.3±11.1 beats/min in the second week (P<0.001) and it fluctuated around this value till the end of the experiment (Fig. 4).

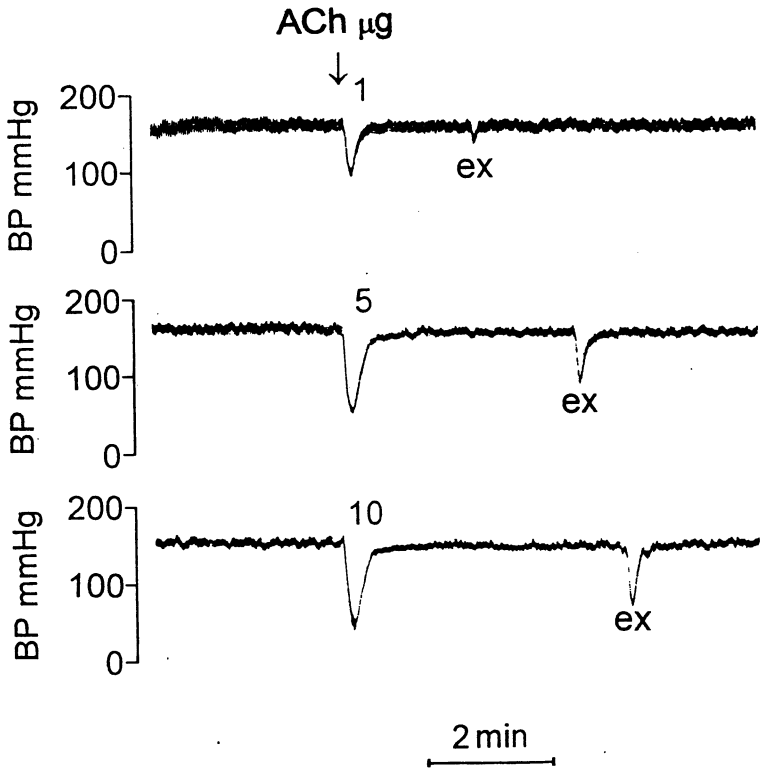

Fig. 5. *Original record of hypotensive response to acetylcholine i.v. (1µg, 5 µg, 10 µg) of a rat which received NO synthase inhibitor L-NAME 50 mg/kg b.w, daily, in drinking water for a period of 6 weeks. Ex indicates cleaning of the cannula by Krebs solution.*

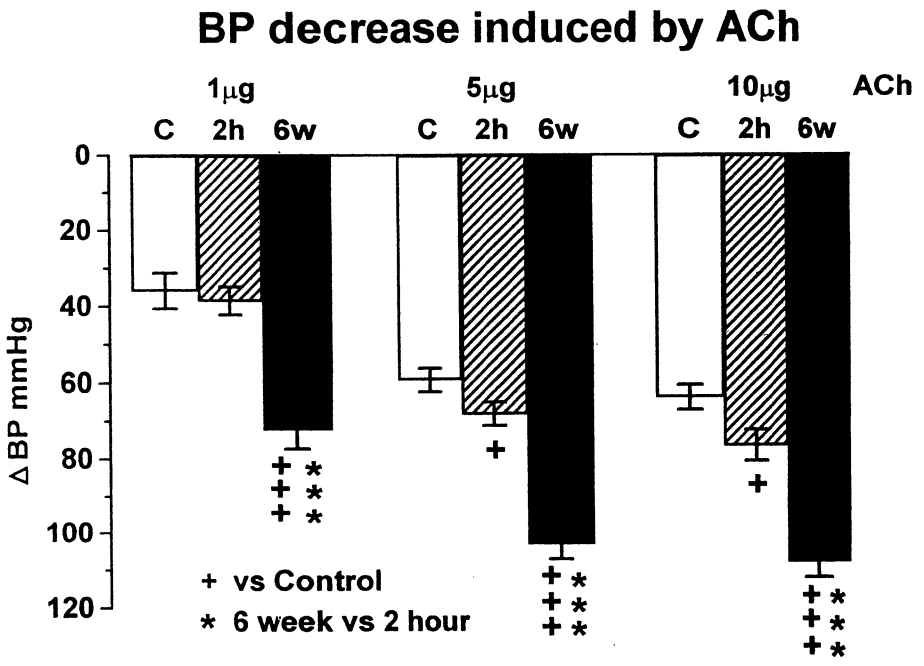

Fig. 6. *Blood pressure decrease in mm Hg induced by acetylcholine i.v. 1 µg, 5 µg, 10 µg/0.1 ml Krebs solution, administered in 10 s: in control rats (open columns), in rats two hours after L-NAME administration (hatched columns), and in rats administered L-NAME (50 mg/kg b.w daily) continually for a period of six weeks (black columns). Significance: + for p<0.05, +++ for p<0.001 and *** for p< 0.001..*

The hypotensive response to acetylcholine in the doses 1 μg, 5 μg and 10 μg was distinctly present in these animals, as demonstrated in the original recordings (Fig. 5). The respective mean values of the hypotensive response to individual doses of 1 μ, 5 μg and 10 μg were 72.4±5.1 mm Hg, 103.4±4.0 mm Hg and 108.3±4.0 mm Hg. All three values were significantly enhanced, not only compared with control values: 35.9±4.7 mm Hg ($P<0.001$), 59.3±3.1 mm Hg ($P<0.001$) and 64.3±3.3 mm Hg ($P<0.001$), respectively, but also with values recorded after two-hour inhibition of NO synthase: 38.5±3.8 mm Hg ($P<0.001$), 68.3±3.1 mm Hg ($P<0.001$) and 76.8±4.1 mm Hg ($P<0.001$), respectively (Fig. 6).

After the blockade of muscarinic receptors by atropine (0.5 mg/kg i.v.), the hypotensive response to acetylcholine disappeared in all 8 animals tested (Fig. 7A).

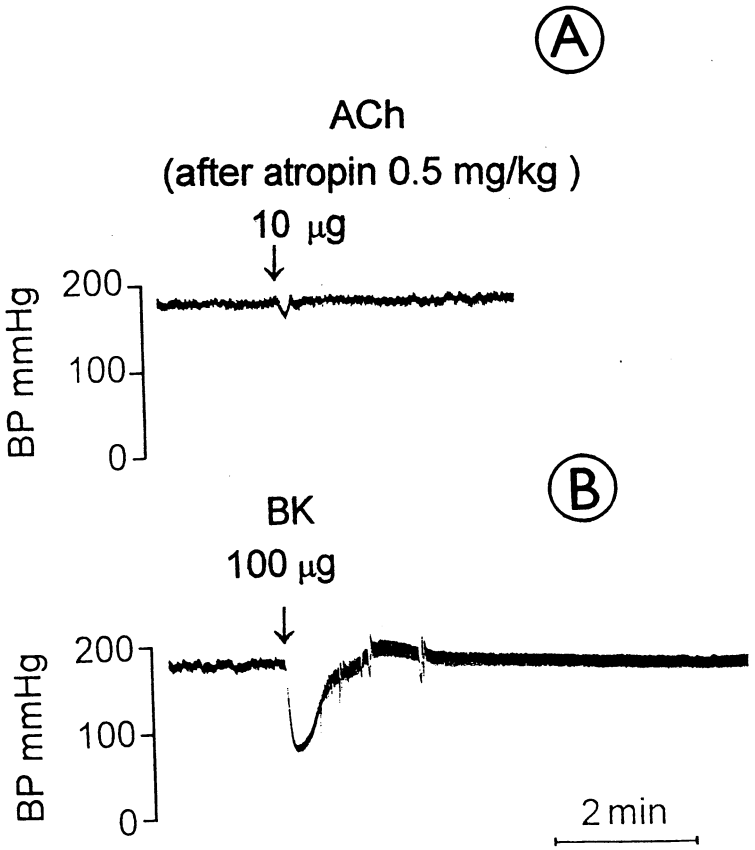

Fig. 7 A. *Original record of blood pressure of a rat administered L-NAME /50 mg/kg b.w. daily) in drinking water for a period of six weeks. Acetylcholine was administered 20 min after atropine 0.5 mg/kg was given i.v.* **B.** *Persisting hypotension response to bradykinin (100 μg/0.1 ml Krebs solution/10 s) in the rat after muscarinic receptors blockade with atropine (0.5 mg/kg).*

On using bradykinin (100 μg) as activator of NO synthase activity, the following hypotensive responses were obtained: 53.3±5.2 mm Hg in the controls, 63.4±7.1 mm Hg two hours after NO synthase inhibition, and 82.8±7.2 mm Hg ($P<0.05$) after 6 weeks of continual NO synthase inhibition (Fig. 8).

The hypotensive response to bradykinin persisted even after the acetylcholine-induced hypotensive response had disappeared due to the muscarinic receptor blockade (Fig. 7B). The hypotensive response to 100 μg bradykinin was 82.8±7.2 mm Hg, and after blockade of muscarinic receptors by atropine, the same dose of bradykinin induced a blood pressure decrease by 81.3±4.7 mm Hg.

Discussion

The present results have proved that the hypotensive response to acetylcholine was present in acute experiments two hours after NO synthase inhibition with L-NAME. The comparison of net differences of blood pressure change induced by acetylcholine in the controls and 2 h after L-NAME administration indicated that the response after inhibition of NO synthase was even

more pronounced. The response to bradykinin tended to increase (but not significantly). The results of our experiments with acetylcholine administered two hours after L-NAME administration are in concert with the findings of Gardiner *et al.* (1990) and Kakizoe *et al.* (1998) who also found a greater hypotensive response to acetylcholine after NO synthase blockade. However, Gardiner *et al.* (1991) described in their next study the same extent of a similar hypotensive response to acetylcholine before and after administration of the NO synthase inhibitor. Contrary to the results of Gardiner *et al.* (1990, 1991) and our present results, Rees *et al.* (1990) found a decrease in response to acetylcholine and bradykinin after L-NAME administration. In their experiments, the inhibition was more marked when bradykinin was used as NO synthase activator.

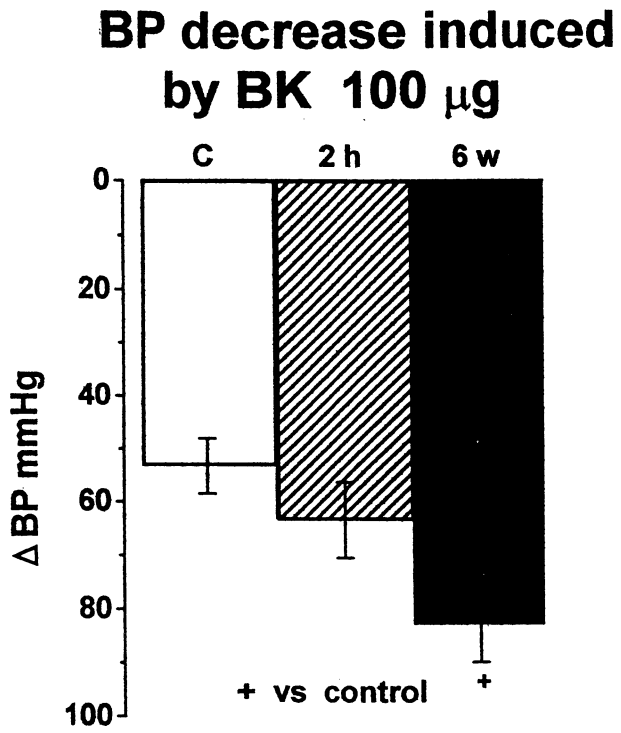

Fig. 8. *Blood pressure decrease in mm Hg induced by bradykinin (100 μg/0.1 ml Krebs solutio) in 10 s in control rats (open columns), in rats two hours after L-NAME was administered (hatched columns), and in rats administered L-NAME (50 mg/kg b.w. daily) continually for a period of six weeks (black columns). Significance + for $p<0.05$.*

The results from acute *in vivo* experiments on anesthetized rats, although diverse, differed significantly from those performed on isolated vessels. The relaxation of isolated vessels to acetylcholine was remarkably attenuated, or even completely inhibited by L-NAME, inhibitor of NO synthase (Rees *et al.* 1990, Török *et al.* 1995).

The main finding of our experiments has provided evidence that the hypotension response to acetylcholine and bradykinin was present in rats with inhibition of NO synthase lasting 6 weeks. The blood pressure in these rats increased and was maintained at a high level throughout the period of 6 weeks. The hypotensive response to acetylcholine, found after six weeks of effective NO synthase inhibition, was significantly larger compared to the response of control age-matched rats with normally operating NO synthase. Moreover, the hypotensive response in the chronically hypertensive rats was also significantly greater, when compared with the response to acetylcholine in rats 2 h after acute administration of L-NAME.

In the animals with chronic inhibition of NO synthase, the hypotensive response to acetylcholine disappeared after i.v. administration of atropine, indicating the involvement of muscarinic receptors. The hypotensive response to bradykinin, however, persisted even after atropine administration and disappearance the response to acetylcholine.

A hypothetical possibility should be considered that the endothelium-derived hyperpolarizing factor is involved (Chen *et al.* 1988, Feletou and Vanhoutte 1988). The hyperpolarizing factor contributes to vascular smooth muscle relaxation after acetylcholine administration. It is conceivable that the hyperpolarizing factor might compensate the missing nitric oxide after inhibition of NO synthase and reduced NO generation from arginine. However, in chronic NO-deficient rats with hypertension and an amplified hypotensive response, a compensatory hyperproduction of the hyperpolarizing factor may be assumed.

According to Folkow's idea (1958), which is still being accepted, the alterations in vessel geometry due to hypertrophy and/or hyperplasia of vascular smooth muscle cells are the essential factor for the development of hypertension. Vessels altered in this way are not able to relax like normal vessels.

Our previous findings appeared to support Folkow's idea at least in large vessels. In NO-deficient hypertensive rats, the wall thickness of coronary and carotid arteries increased significantly (Kristek *et al.* 1996). Thus, similar structural alterations may be found even in resistance vessels.

Folkow's idea (1958) and the above considerations are at variance with the suggestion of

Haegerty *et al.* (1993) who explained the increase of wall thickness in hypertension in general as a remodeling of the structural relations among individual components of the vessel wall, without proper hypertrophy and/or hyperplasia of smooth muscle cells in the media and also without an increase in the content of non-cellular components. The relaxation of smooth muscle cells after acetylcholine administration should lead to a wall/lumen ratio close to that of control animals. Nevertheless, to accept the idea of Haegerty *et al.* (1993) it would be necessary to measure not only the wall thickness of resistant arteries but also to assess the intimate architecture of smooth muscle cells and their relation to elastic and collagen fibers in crucial vascular areas.

The compromised function of the heart after NO synthase inhibition may contribute to the enhanced hypotensive response induced by acetylcholine. Actually, Le Chevalier *et al.* (1994) reported a decrease in cardiac output in anesthetized dogs immediately after administration of L-NAME. Babál *et al.* (1997) found frequent small fibrotic areas in the myocardium in NO deficiency. Pecháňová *et al.* (1997) described an increase of non-contractile proteins in the myocardium induced by increased blood pressure in chronic inhibition of NO synthase. On the other hand, in our morphometric study (Sládek *et al.* 1996), we found an increase in the cross-section area of myocytes indicating hypertrophy of cardiac myocytes at least in the anterior wall of the left ventricle. Thus, some areas of compensatory hypetrophic myocytes may be found in the myocardium during long-term NO deficiency.

The above findings indicate that the myocardium and its blood supply require a certain level of nitric oxide for the maintenance of normal working efficacy. After inhibition of NO generation, the structure and function of the myocardium is probably compromised. This idea is supported by the recent experiments of Kojda *et al.* (1997).

In conclusion, the hypotensive response to acetylcholine is significantly enhanced in rats exposed for 2 h and particularly for six weeks continually to NO synthase inhibition by L-NAME. After blockade of muscarinic receptors, the hypotensive response disappeared. However, the hypotensive response to the other activator, bradykinin, persisted even after 2 h and was still enhanced after six weeks of NO synthase inhibition.

The experiments do not allow to recognize the actual mechanisms underlying this described phenomenon. Nevertheless, they undermine the simple reductionistic idea: the activation of NO synthase triggers and/or the inhibition of NO synthase inhibits the reaction arginine → citrulline + NO → vascular smooth muscle relaxation. Further experiments are needed to understand the interference of NO in the manifold diversified control mechanisms of the cardiovascular system.

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Reprint requests

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