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VASODILATORY PROPERTIES OF MONO-L-ARGININE-CONTAINING COMPOUNDS

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SUMMARY: Benzoyl derivatives of L-arginine, unlike arginine, elicited relaxation of pre-contracted rat aortic rings in a concentration dependent manner. The most potent relaxing agent was N-alpha-benzoyl-L-arginine ethyl ester. The relaxation was abolished by methylene blue, but not by indomethacin. When incubated with rat aortic rings, the benzoyl derivatives exhibited colorimetric reactions characteristic of citrulline and nitrite ion. This indicates the presence of a peptidyl arginine deiminase like activity in rat aorta. Citrulline had no vasodilatory property. The other product of the iminase reaction is ammonia which through oxygenase pathway may generate nitric oxide, the proposed endothelium derived relaxing factor(EDRF). Our results suggest that an as yet unidentified arginine derivative from the endothelium may be the biological precursor of EDRF.

The obligatory role of vascular endothelium to evoke relaxation of the underlying smooth muscle was first reported by Furchgott and Zawadzki in 1980 (1). Since then, many of the vasodilatory agents are shown to elicit smooth muscle relaxation by releasing a factor, the endothelium derived relaxing factor (EDRF). Recent studies show that the pharmacological properties of EDRF and nitric oxide (NO) are remarkably similar (2,3). Palmer et al (4) report that cultured endothelial cells release NO when challenged with either bradykinin or the calcium ionophore A23187. Based on these experimental findings, many investigators conclude that EDRF is NO (2-4). However, at present no information is available regarding the biological source of NO or the sequence of events in the biosynthesis of NO.

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Recently, we reported that several peptides which elicit endothelium dependent relaxation possess L-arginine in their amino acid sequence (5). However, by itself, L-arginine has no vasodilatory property. Here we report that benzoyl derivatives of arginine have vasodilatory properties. We also provide evidence for the existance of a peptidyl arginine deiminase pathway in the vascular wall and propose for the first time an enzymatic mechanism for the generation of NO from arginine-containing peptides.

METHODS

N-alpha-benzoyl-L-arginine, N-alpha-benzoyl-L-arginine ethyl ester, L-arginine ethyl ester, homoarginine, L-arginine, citrulline and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO).

Aortic rings (3-4mm) were prepared from male Sprague Dawley rats (250-300 g) as described previously (5). The rings were suspended in 10 ml organ chambers under an optimal tension of 1.5 g and the tension was monitored with a Harvard (Model 363) force transducer. The rings were maintained at 37^0C in Krebs bicarbonate buffer (pH 7.4) of the following composition (mM); NaCl,116; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 23; and glucose, 11. The solution was continuously gassed with 95% O_2 and 5% CO_2. The rings were equilibrated for an hour, during which time the buffer was changed every 15 min. After the equilibration period, the rings were contracted with phenylephrine (1×10^{-7}M) and cumulative dose response curves were obtained for each of the benzoyl derivatives of arginine and also for L-arginine ethyl ester, homoarginine and arginine. The soluble guanylate cyclase inhibitor, methylene blue, and the cyclo-oxygenase inhibitor, indomethacin, were added to the bath 15 to 20 min. prior to contracting with phenylephrine. In some preparations, the endothelium was removed by gently rotating a metal spatula through the lumen of the aorta and the efficacy of the endothelium removal was confirmed by the absence of relaxation to acetylcholine.

The formation of citrulline was followed by using the colorimetric procedure described by Rothnagel and Rogers (6). The color developed was measured at 530 nm. Nitrite content was measured according to the method of Green et al (7) by the diazo reaction (measured at 548 nm).

RESULTS AND DISCUSSION

The effect of N-alpha-benzoyl-L-arginine ethyl ester on rat aortic rings pre-contracted with phenylephrine is shown in figure 1. Relaxation was observed in rings with and without endothelium. However, rings with endothelium were more sensitive.

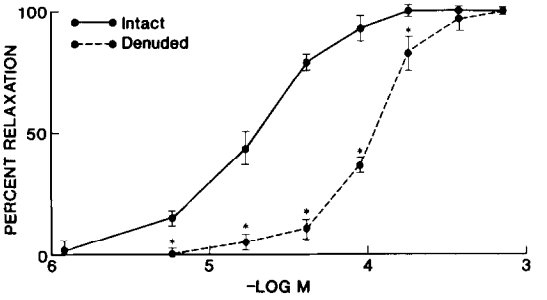

Fig. 1. Effect of N-alpha-benzoyl-L-arginine ethyl ester on intact and denuded rat aortic rings pre-contracted with phenylephrine (1×10^{-7}M). Values are mean ± S.E.M. of 8 experiments *P<.05.

The potency of relaxation (EC_{50}, the concentration for 50% relaxation) of several benzoyl derivatives of L-arginine, L-arginine ethyl ester, homoarginine, D- and L-arginine are shown in table 1. For all the arginine derivatives, the aortic rings with endothelium were more sensitive than those without endothelium. Homoarginine, D- and L-arginine have no vasodilatory effect whether endothelium was present or not. N-alpha benzoyl-L-arginine ethyl ester was the most potent relaxing agent, both in presence and absence of endothelium.

TABLE 1

EFFECT OF MONO L-ARGININE COMPOUNDS ON RAT AORTIC RINGS WITH (+) AND WITHOUT ENDOTHELIUM (-)

COMPOUND	$EC_{50} \times 10^{-5}$ M ENDOTHELIUM	
	+	-
N-alpha benzoyl L-arginine ethyl ester	2.0 ± .31	10.0 ± .26
Benzoyl L-arginine	10.4 ± .18	171.0 ± .53
Arginine ethyl ester	10.2 ± .20	26.0 ± .41
Homo L-arginine	no effect	no effect
L-Arginine	no effect	no effect
D-Arginine	no effect	no effect

EC_{50}, Molar concentration of agonist producing 50% of maximal response (expressed as percent of phenylephrine (1×10^{-7}M) induced contraction. Results are mean ± S.E.M of 8 determinations.

Figure 2 shows the effects of indomethacin (2×10^{-6} M) and methylene blue (1×10^{-5} M) on the relaxation. It is clear that the benzoyl derivative of arginine elicits vascular relaxation by activating soluble guanylate cyclase and not by releasing vasodilatory prostaglandins such as prostacyclin. Similar responses were observed with the other benzoyl derivatives and the ethyl ester of arginine.

Several studies show that the endothelium dependent relaxation is closely associated with the activation of soluble guanylate cyclase in smooth muscle (8,9). Deguchi and Yoshioka report that in neuroblastoma cells, L-arginine activates soluble guanylate cyclase, but D-arginine or other basic amino acids are ineffective (10). In addition, they report that several peptides possessing L-arginine in their sequence, either at the N-terminal or the C-terminal, also activate soluble guanylate cyclase. Interestingly, we observed that several of the peptides, which elicit endothelium dependent relaxation, also contain arginine near/or at the C- or N-terminal (5). In addition, we reported that basic poly peptides such as poly L-arginine, poly L-lysine and mellitin (a bee venom peptide) also elicit endothelium dependent relaxation. However, the basic amino acids (L-arg, L-lys and L-gln), by themselves have no vasodilatory property. Hence L-arg is not the EDRF. This is not surprising since,

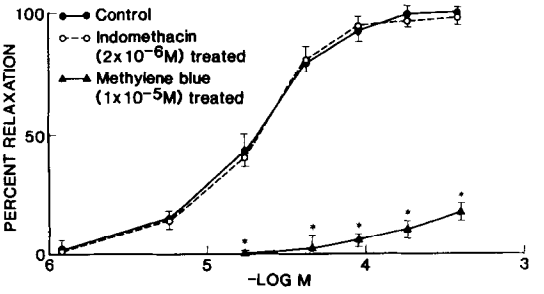

Fig. 2. Effects of cyclo-oxygenase inhibitor, indomethacin, and soluble guanylate cyclase inhibitor, methylene blue, on the relaxation response of N-alpha-benzoyl-L-arginine ethyl ester in rat aortic rings. Values are mean ± S.E.M. of 6 experiments. *P<.05.

EDRF has a half life of 6-50 secs (11) whereas, arginine is quite stable. Comparative pharmacological and chemical studies indicate remarkable similarities between EDRF and nitric oxide (NO) (2-4). Eventhough other investigators have raised doubts (12) about the identity of EDRF as NO, the current thinking is that EDRF is either NO, or a closely related compound. However, at present no information is avilable regarding the biological source of NO.

Hibbs et al (13) have shown that activated mouse macrophages synthesize L-citrulline and nitrite (NO_2^-) when the medium is supplemented with L-arginine and this is not observed with any other amino acids including D-arginine. From these studies, they proposed the existance of a deiminase pathway in mouse macrophage, which generates L-citrulline and ammonia from arginine. Oxidation of ammonia by oxygenase generates nitrite, possibly via the intermediate formation of NO. However, in our experiments, incubation of rat aortic rings with L-arginine failed to generate any citrulline or nitrite (presence of which were tested by the colorimetric tests described in the materials and methods section). On the other hand, when rat aortic rings were incubated with the benzoyl form of arginine, we observed the colorimetric reactions for both citrulline and nitrite formation (data not shown). Citrulline does not possess vasodilatory properties whereas, nitrite does. This raises the interesting possibility that vascular endothelium and possibly smooth muscle have a deiminase activity. Past studies have shown the existance of peptidyl arginine deiminase in the epidermis of newborn rats (14) and rabbit skeletal muscle (15). This iminase by acting upon arginine-containing peptides as well as N-substituted arginine derivatives, generates citrulline. In rabbit skeletal muscle, for the N-substituted compounds the highest iminase activity is found for N-alpha-benzoyl-L-arginine ethyl ester (15). Interestingly, this

benzoyl derivative had the highest relaxing activity in our bio-assay. Peptidyl arginine deiminase has no activity towards L-arginine. This supports our previous report that peptides elicit endothelium dependent relaxation due to the presence of L-arginine in their sequence (5). It is possible that the other non-peptide EDRF generating agents such as acetylcholine may also release arginine containing peptides from the endothelium. The half-life of the released arginine containing peptides may be extremely small *in vivo*. Studies by Bachmair *et al* (16) have shown that *in vivo* half-life of the enzyme beta-galactosidase is dramatically reduced from more than 20 hours to less than 3 min when an arginine is attached to its amino-terminus (the "N-end rule").

Thus, we report here for the first time the possible existance of a peptidyl arginine deiminase pathway in vascular endothelium (and may be also in the smooth muscle) which from arginine containing peptides generates citrulline and ammonia. Ammonia up on oxidation yields NO. However, so far, the formation NO or NO_2^- from ammonia by the oxygenase pathway has not been shown in any mammalian systems, eventhough *in vivo* formation of nitrate (NO_3^-) from ammonia is reported in man (17) and rat (18). Martin *et al* (19), recently report the presence of nitrite in bovine endothelial cells which upon acidification (pH 2) yields NO and they speculate the presence of a nitrate reductase in the endothelial cells. Eventhough it is an attractive hypothesis, it may be unlikely since, nitrate reductases which are reported so far only in bacterial systems are inactive in presence of oxygen. Hence, a likely pathway for the generation of NO (EDRF) is the peptidyl arginine deiminase acting on an arginine containing moiety from the endothelium, and the ammonia generated in this step is converted to NO, probably by the action of oxygenases as reported for the mouse macrophages(13).

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