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Vascular Effects of L-Arginine: Anything beyond a Substrate for the NO-Synthase?

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L-arginine supplementation is hypothesized to reduce endothelial dysfunction and atherogenesis via increased biosynthesis of nitric oxide. Here we describe superoxide scavenging properties of arginine as an additional aspect which needs to be considered. Furthermore, arginine reduced copper-induced lipid peroxidation, indicating that superoxide anions essentially contribute to this process. In intact endothelial cells, L-arginine but not D-arginine diminished superoxide release and reduced cell-mediated breakdown of nitric oxide. Our data indicate that the reported vascular effects of L-arginine supplementation might involve an increased bioavailability of nitric oxide due to its superoxide scavenging properties beside a potential increased NO biosynthesis.

L-arginine has been shown to reestablish endothelium-dependent vasodilation in animal models (1, 2) and humans (3) with endothelial dysfunction (4, 5). Furthermore, supplementation with exogenous L-arginine has been shown to constitute anti-atherosclerotic effects in experimental animals (6, 7) Endothelial dysfunction has been shown to be based on a reduced formation or bioavailability of nitric oxide (NO) in various diseases (8). Since L-arginine is the substrate for the NO-synthase (for review see (9, 10)) the current hypothesis is that L-arginine exerts its effects via increased formation of NO in vascular endothelial cells. This hypothesis neglects that such effects of L-arginine could be found in humans without any evidence of L-arginine deficiency (3). Furthermore, intracellular concentration of L-arginine is about 500-800 μM (11) and thus far above the K_m for the NO-synthase (4 μM; (12)). Moreover, endothelium-dependent relaxation is completely blunted in blood vessels that are L-arginine depleted to 30% of its normal value (i.e. 90 nmol/g tissue; (13)), although the remaining L-arginine concentration should still serve as substrate for the NO-synthase. These controversies may indicate that L-arginine beside serving as substrate for NO-synthase has additional effects on vascular reactivity. Since antioxidants, such as vitamin E and vitamin C have been shown to reduce atherogenesis in primates (14) and to ameliorate endothelial dysfunction in humans (8), similar to L-arginine, this study was designed to investigate whether anti-oxidative properties of L-arginine might contribute to its beneficial effect on the vascular system.

MATERIALS AND METHODS

Materials. All materials unless indicated were purchased from Sigma Chemicals (Vienna, Austria).

Measurement of pyrogallol autooxidation. Pyrogallol autooxidation was measured as described for assessment of SOD activity (15). Briefly, 40 μl of the substance solution to be tested and 100 μl of Tris-HCl-EDTA Buffer (1M Tris-HCl and 5 mM EDTA, pH 8 with 0.1 N NaOH) were added to 840 μl of water in a cuvette. Cuvettes were placed in a photometer (Shimadzu RF 160A) at 25° Celsius. Aminoacids (up to 4 mM final concentration) were dissolved in Tris-HCl-EDTA Buffer to avoid changes of pH in the assay. Reaction was started by addition of 20 μl pyrogallol (10 mM in 10 mM HCl) under short steering. The change of absorption at a wavelength of 320 nm was monitored for 3 minutes. Results of 4 to 6 experiments are given as change of absorption per minute (mean ± standard deviation).

Enzymatic generation of superoxide anions. Superoxide anions were generated by the reaction of xanthine oxidase (150 μU/ml) with hypoxanthine (1 mM) in HEPES-buffer containing 30 μM Ca^{2+} at 37°C as described previously (16).

Cell culture. Endothelial cells isolated from porcine aortae were used. Cells were cultured in Opti Dulbecco minimal essential medium (Gibco) containing 3% fetal calf serum (PAA, Linz, Austria). For experiments, cells were seeded in 6 well plastic dishes or in petri dishes (10 cm diameter). All experiments were performed at 37°C with cells up to passage 1.

Measurement of superoxide generation. Superoxide anions were determined as the reduction of ferricytochrome C (16). Cultured cells were incubated with Hepes-buffered solution containing ferricytochrome C (10 μmol/l; horse heart type III) in the absence or presence of SOD (476 U/ml) and the reduction of ferricytochrome C was fol-

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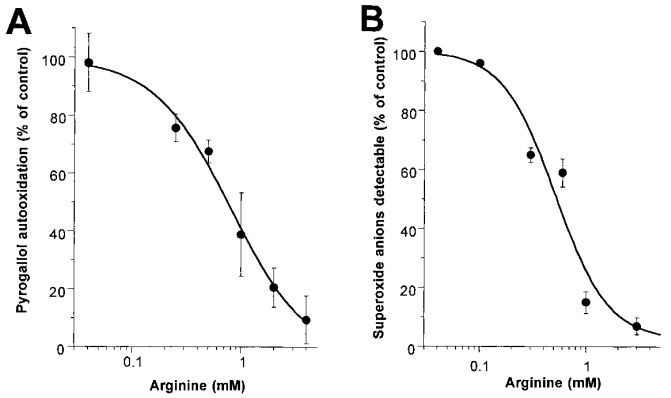

FIG. 1. The effect of arginine on superoxide anion-mediated pyrogallol autooxidation (panel A) and ferricytochrome c reduction (panel B). A: Concentration-dependent reduction of pyrogallol autooxidation (expressed as % of control) by L-arginine determined photometrically at 320 nm. B: Superoxide anions were generated by the hypoxanthine/xanthine oxidase reaction in the presence of given arginine concentration. The amount of free superoxide anions was estimated by the reduction of ferricytochrome c measured at 550 nm. The results were expressed as percentage of ferricytochrome c reduction in the absence of arginine. Each point represents the mean±SEM (n=4–6).

lowed at 550 nm. Differences in the extinction in the absence and the presence of SOD represent superoxide anion related reduction of ferricytochrome C and can be calculated using the molar extinction coefficient of reduced form of ferricytochrome C (ϵ=21.000).

Measurement of lipid oxidation. Oxidation of Serum lipids was continuously measured by a fluorescence method as recently published (17). Argon saturated serum of healthy donors was incubated for 12 hours at 37°C under argon with 1-palmitoyl-2-((2-(4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl)ethyl)-carbonyl)-*sn*-glycero-3-phosphocholine (DPHPC, 20 nmol/ml serum). For measurements 30μl aliquot is diluted with 2.655 ml PBS, 300 μl of aminoacid solutions (in PBS) were added prior the experiments to the final concentrations mentioned. Oxidation is started by addition of 15 μl of a 10 mM $CuSO_4$ solution. Oxidation is monitored by the time dependent decrease in fluorescence intensity at 430 nm (excitation at 360 nm) with a Hitachi F2000 fluorometer. Results represent the mean ± standard deviation of 4 experiments for each concentration.

Measurement of NO degradation. Degradation of nitric oxide was measured using a porphyrinic-based sensor (ISO-NO; WPI, Berlin, Germany) as describe by Malinski and Taha (18) Saturated nitric oxide solution was added to the solution containing the L-arginine concentration indicated and degradation of nitric oxide was followed by the sensor.

Statistics. All results are expressed as mean values ± S.E.M. Statistical significance was evaluated using Anova including Tukey HSD for intergroup differences. Level of significance was defined as $P<0.05$ in all experiments.

RESULTS

In the presence of L-arginine, autoxidation of pyrogallol was diminished in a concentration dependent manner (Fig.1A). Thereby, pyrogallol autooxidation was significantly reduced in the presence of physiological intracellular concentrations of L-arginine (i.e. 0.8±0.2 μM; (11)), while EDTA had no effect (data not shown). In agreement with these findings, L-arginine attenuated reduction of ferricytochrome c by superoxide anions, generated by the reaction of xanthine oxidase with hypoxanthine (Fig.1B). In both experimental setups, D-arginine proved to be equally effective than L-arginine (data not shown).

These putative superoxide-anion-scavenging properties of arginine affected copper-induced oxidation of plasma lipoproteins (Fig.2): the initial slow propagation phase remained unaffected in the presence of various concentrations of L-arginine. However, the steep propagation rate of lipoprotein oxidation was reduced from 3.42±0.41 % F.I./min in the absence of L-arginine to 1.09±0.33 and 0.43±0.22 % F.I./min in 0.5 and 1.0 mM L-arginine, respectively (n=4, $P<0.001$; inset Fig.2). Identical results were obtained using D-arginine (inset Fig.2). Similar to arginine, superoxide dismutase reduced the steep propagation rate of copper-initiated lipoprotein oxidation (inset Fig.2).

To investigate a possible physiological importance of the reported superoxide anion scavenging properties of arginine, the effect of a preincubation with L-/D-arginine on superoxide anions release of porcine aortic endothelial cells were investigated. Incubation of cells with L-arginine diminished the amount of superoxide anion detectable in a concentration dependent manner (Fig.3A). In contrast, incubation with D-arginine did not affect superoxide anions release (Fig.3A). In agreement to its inhibitory properties on the amount of superoxide anions derived from endothelial cells, L-arginine protected extracellularly applied NO from degradation by cell-derived superoxide anions (Fig.3B).

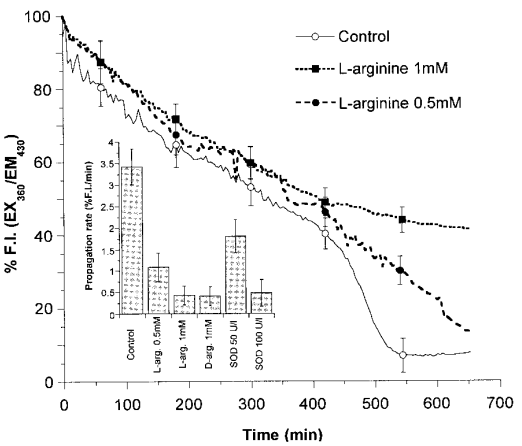

FIG. 2. Effects of two L-arginine concentrations (0.5 and 1 mM) on copper-induced oxidation of 1-palmitoyl-2-((2-(4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl)ethyl)-carbonyl)-*sn*-glycero-3-phosphocholine (DPHPC) in the surface of plasma lipoproteins from healthy donors (n=4). Oxidation process was monitored by the reduction of the fluorescence intensity (F.I.) at 360 nm excitation and 430 nm emission. The inset shows the steep propagation rate expressed as % change of F.I./min in the absence (control) or presence of L-/D-arginine and SOD.

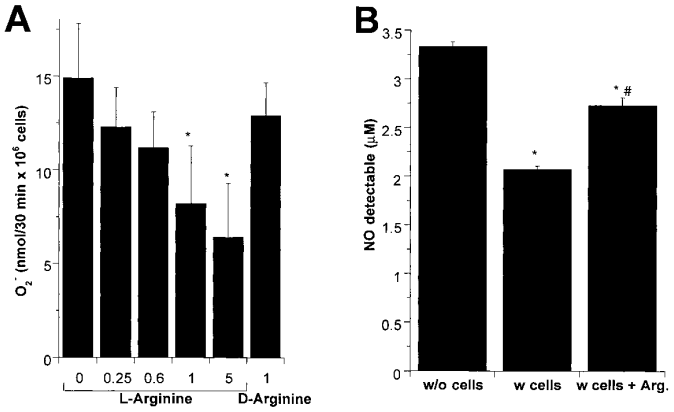

FIG. 3. Effect of the arginine isomers (D,L) on superoxide anion release of endothelial cells (panel A) and cell-mediated degradation of extracellularly applied nitric oxide (panel B). Confluent endothelial cells were incubated for 24 h in arginine-free minimal essential medium. Just prior the experiments cells were harvested by enzymatic digestion, centrifuged and resuspended in HEPES-buffered solution (2.5×10^6 cell/ml) containing the arginine concentration indicated. A: Release of superoxide anions was measured by the reduction of ferricytochrome c at 550 nm. B: A given amount of nitric oxide (NO; 3.5 μM) was applied to the cell suspension in the absence and presence of 1 mM L-arginine and NO degradation was monitored using a porphyrinic-based NO sensor. Each column represents the mean±SEM (n=6-12). *$P<0.001$ vs. without cells. #$P<0.01$ vs. with cells in the absence of L-arginine.

DISCUSSION

The results presented indicate by two different experimental approaches the ability of L-/D-arginine to scavenge superoxide anions. Pyrogallol, in the presence of oxygen undergoes spontaneous autooxidation and yields colored autooxidation-compounds. During the autooxidation O_2^- is generated and thus pyrogallol can be used as an O_2^- donor (19, 20). Since, generation of O_2^- itself catalyses the autooxidation process, this reaction was initially used for determination of SOD activity (15). Thus, substances detoxifying the reactive oxygen species O_2^- reduce the formation of the colored reaction products. Transition metal chelators such as EDTA on the other hand have only minor effects in the system used, indicating the small role this metals have in the autooxidation of pyrogallol. These findings were confirmed by the effect of L-/D-arginine on reduction of ferricytochrome c by enzymatically generated superoxide anions (xanthine oxidase/hypoxanthine reaction). Thus, these data indicate that independent of its steric isoform form, the amino acid arginine is able to scavenge superoxide anions.

As a consequence of this free radical scavenging property of arginine, the copper-initiated lipoprotein oxidation could also be diminished by arginine. In general, a two phase oxidation of plasma lipoproteins under low radical flux conditions can be observed (for review see:(21)). Interestingly, arginine only affected the later, steep propagation rate of lipoprotein oxidation, while the initial, slow oxidation rate remained unaffected by arginine. Similar results were obtained using superoxide dismutase indicating that superoxide anions contribute to the steep propagation rate, while an involvement of this type of free radical in the initial slow oxidation rate seems unlikely. The potency of L-arginine to prevent lipoprotein oxidation has been shown recently (22). Our results demonstrate that this anti-oxidative property of arginine on lipoprotein oxidation is due to its superoxide scavenging effect.

A potential physiological role of these results were indicated by our findings that L-arginine was also able to diminish basal superoxide anion release from cultured endothelial cells and aortic rings from hypercholesterolemic rabbits (23). Endothelial superoxide anion formation has been shown to be increased under numerous pathological conditions, such as hyperglycemia (24) or hypercholesterolemia (23). Convincingly, endothelial-initiated degradation of extracellularly applied NO was found to be reduced when cells were preincubated with L-arginine. These data indicate that L-arginine might be able to increase the bioavailability of NO, due to a reduction of superoxide anion release resulting in a reduced extracellular breakdown of NO. In contrast to the experiments using the isolated superoxide anion generating systems (e.g. pyrogallol, xanthine oxidase/hypoxanthine) in intact cells only L-arginine proofed to be effective. These findings are consistent with the lack of D-arginine to influence endothelium-dependent relaxation in humans (25) while L-arginine (partially) normalizes endothelial function. It has been shown that basic amino acid transporter (Y^+) in endothelial cells is stereoselective and prefers L-arginine to D-arginine by about 100 times (26). This might, in our opinion, explain the stereoselective effect of arginine in intact cells/animals. However, very high concentrations of D-arginine have been shown to induce vasodilation in humans (27) and to reduce blood pressure in rats (28). Since D-arginine does not serve as a substrate for endothelial NO synthase, these data suggest protection of NO by D-arginine either extracellularly and/or within the cells due to uptake of the D-arginine present in highly excessive concentrations.

Classical antioxidants have been shown to reduce atherosclerosis in experimental models (14, 29), to exert hypotensive effects (30) and to improve insulin sensitivity in humans (31-33). These properties have also been shown for L-arginine (3, 34-36).

So far different pathways have been suggested for the molecular mechanisms behind these effects of the antioxidants and L-arginine (free radical scavenging and increased NO biosynthesis, respectively). Our data presented here provide new evidence that besides its potential effect on NO biosynthesis, the beneficial properties of L-arginine on the vascular sys-

tem involves scavenging of superoxide anions and sparing effects of NO.

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