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Homocysteine, a risk factor for atherosclerosis, biphasically changes the endothelial production of kynurenic acid

Janusz Stazka[a], Piotr Luchowski[a,b], Ewa M. Urbanska[b,c,*]

[a]Department of Cardiosurgery, Skubiszewski Medical University, Jaczewskiego 8, 20-090 Lublin, Poland
[b]Department of Pharmacology and Toxicology, Skubiszewski Medical University, Jaczewskiego 8, 20-090 Lublin, Poland
[c]Department of Toxicology, Institute of Agricultural Medicine, Jaczewskiego 2, 20-950, Lublin, Poland

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Abstract

Increased serum level of homocysteine is an independent risk factor for vascular disease. The effect of DL-homocysteine on the endothelial production of kynurenic acid, an antagonist of α7-nicotinic and *N*-methyl-D-aspartate (NMDA) glutamate receptors, has been evaluated in vitro and in vivo. In rat aortic rings, DL-homocysteine at 40–100 μM enhanced, whereas at ≥400 μM decreased the synthesis of kynurenic acid. *S*-adenosylhomocysteine mimicked the biphasic action of DL-homocysteine. On the contrary, thiol-containing compounds, L-cysteine and L-methionine, were only inhibiting kynurenic acid production. L-kynurenine uptake blockers, L-phenylalanine and L-leucine, reversed the stimulatory effect of *S*-adenosylhomocysteine. L-glycine, co-agonist of NMDA receptor, and *cis*-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755), an antagonist of NMDA receptor, have not influenced kynurenic acid formation. In vivo, DL-homocysteine (1.3 mmol, i.p.) increased the level of kynurenic acid in rat serum from 23.7±7.1 to 60.7±14.2 (15 min, $P<0.01$) and 55.7±13.6 (60 min, $P<0.01$) pmol/ml, respectively; the endothelial content of kynurenic acid was also increased (51.6±5.8 vs. 73.2±9.4 fmol/μg of protein; 15 min; $P<0.01$). DL-homocysteine seems to modulate the production of kynurenic acid both directly and indirectly, possibly following the conversion to *S*-adenosylhomocysteine. The obtained data suggest a potential contribution of altered formation of kynurenic acid to the endothelial changes induced by hyperhomocysteinemia.

Keywords: Endothelium; Homocysteine; Kynurenic acid; *S*-adenosylhomocysteine

1. Introduction

Kynurenic acid, found in the brain and in the periphery, is a product of irreversible transamination of L-kynurenine (Stone, 2001). It is known as an endogenous glutamate receptors antagonist that displays the highest affinity for the strychnine-insensitive glycine site of *N*-methyl-D-aspartate (NMDA) receptor complex (Stone, 2001). Kynurenic acid appears to be produced by various forms of aminotransferases which, depending on the tissue and animal species, differ as regards their biochemical characteristic (Baran et al., 1997; Buchli et al., 1995; Noguchi et al., 1975). A number of factors regulating the brain synthesis of kynurenic acid has been identified. Mitochondrial toxins impairing oxidative phosphorylation, metabotropic glutamate receptors agonists or the endogenous sulphur-containing amino acids, such as L-cysteine sulphinate or L-homocysteine sulphinate, were shown to reduce kynurenic acid production (Kocki et al., 2003; Luchowski et al., 2002; Urbanska et al., 1997). In contrast, few compounds may increase production of kynurenic acid within the brain, e.g. immunosuppressant FK506 or antiepileptic carbamazepine (Kocki et al., 2004; Luchowska et al., 2003).

Experimentally applied kynurenic acid exerts potent neuroprotective and anticonvulsant properties, attributed mainly to the blockade of glutamate receptors (Stone, 2001). Accumulated evidence seems to support the hypoth-

* Corresponding author. Department of Pharmacology and Toxicology, Skubiszewski Medical University, Jaczewskiego 8, 20-090 Lublin, Poland. Tel.: +48 81 740 5851; fax: +48 81 532 8903.
E-mail address: emurbanska@poczta.onet.pl (E.M. Urbanska).

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esis, formulated over two decades ago, linking the central deficiency of kynurenic acid with neuronal loss and seizures (Stone, 2001). In fact, reduced brain synthesis of kynurenic acid in rodents may cause the neuropathological and behavioral changes resembling Huntington's disease or can induce clonic seizures (Turski et al., 1991; Urbanska et al., 1991). On the other hand, local increase of kynurenic acid content within the brain might be associated with the impairment of cognitive processes or with the development of affective diseases (Baran et al., 1999; Schwarcz et al., 2001).

Kynurenic acid was also demonstrated to block cholinergic α7-nicotinic receptors (Hilmas et al., 2001). Nicotinic receptors are widely distributed and occur on neuronal and non-neuronal cells of different organs, including cardiovascular system. Increasing data suggest that the pathogenesis of neurological and cardiovascular disorders may involve altered function of nicotinic receptors (Pereira et al., 2002; Wang et al., 2001; Wessler et al., 1998).

The role played by kynurenic acid in the periphery is not recognized. Kidneys, liver and heart may all synthesize kynurenic acid (Baran et al., 1997; Buchli et al., 1995; Noguchi et al., 1975). We have shown that vascular endothelium is also able to produce and liberate substantial amounts of kynurenic acid and that hypoglycemic and anoxic conditions may reduce its formation (Stazka et al., 2002), but the contribution of kynurenic acid to vascular physiology and pathology requires further intense studies.

Homocysteine is a methionine-derived sulphur-containing amino acid detected in the human serum. Its increased level is an independent risk factor for vascular disease, possibly also for cognitive dysfunction and psychiatric disorders (Durand et al., 2001; Medina et al., 2001). Hyperhomocysteinemia may stimulate various processes leading to the endothelial cell damage and to a vascular inflammatory response (Dudman et al., 1991; Medina et al., 2001; Poddar et al., 2001). Reduction of endothelial antithrombotic activity, release of proinflammatory cytokines and various growth factors, decreased NO-mediated vasorelaxation, stimulation of smooth muscle cell proliferation or enhancement of collagen production might all occur during hyperhomocysteinemia (Faraci, 2003; Medina et al., 2001; Weiss et al., 2002). Recently, the dual modulation of brain kynurenic acid production displayed by DL-homocysteine has been revealed (Luchowska et al., 2005). Therefore, we decided to investigate the effects of DL-homocysteine and its related metabolites on the endothelium-specific, aortic production of kynurenic acid.

2. Materials and methods

2.1. Animals and substances

Experiments were performed on male Wistar rats (220–250 g) that were housed under standard laboratory conditions. L-kynurenine (sulphate salt), kynurenic acid, DL-homocysteine, *S*-adenosylhomocysteine, L-cysteine, L-methionine, L-glycine, *cis*-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755), L-phenylalanine, L-leucine and other used chemicals were obtained from Sigma-Aldrich Chemicals. All the high pressure liquid chromatography (HPLC) reagents were purchased from J.T. Baker Laboratory Chemicals. All the experimental procedures have been approved by the Local Ethical Committee in Lublin and are in agreement with the European Communities Council Directive on the use of animals in experimental studies.

2.2. Aortic rings

The preparation of aortic rings was performed as described before (Stazka et al., 2002). Following decapitation, the aorta was perfused transcardially with 20 ml of ice-cold oxygenated (95% of O_2 and 5% of CO_2) Krebs–Ringer buffer, pH 7.4, containing 118.5 mM NaCl, 4.75 mM KCl, 1.77 mM $CaCl_2$, 1.18 mM $MgSO_4$, 12.9 mM NaH_2PO_4, 3 mM Na_2HPO_4 and 5 mM glucose. Thoracic portions of aortas were harvested and immediately submerged in an ice-cold Krebs–Ringer buffer. The adhering perivascular tissue was carefully removed. Special care was taken to avoid damage to the endothelium. Aortic rings of 750 μm thickness (McIlwain Tissue Chopper) were randomly transferred into the incubation wells (5 slices per well) containing Krebs–Ringer buffer. As described before (Stazka et al., 2002), the "no endothelium" blanks included use of the endothelium-denuded rings. The endothelial layer was gently removed from the thoracic portion of aorta by rubbing the intimal surface.

2.3. Production of kynurenic acid in vitro

Measurement of kynurenic acid production was performed as described before (Stazka et al., 2002). The aortic rings were incubated (37 °C; 120 min) with tested compounds at various concentrations and with L-kynurenine (final concentration 50 μM). Following the incubation period, the wells were transferred into an ice-cold water bath, media were rapidly separated from the tissue, acidified with 0.1 ml of 1 N HCl and 14 μl of 50% trichloroacetic acid (wt:vol), and centrifuged. Obtained supernatant was stored (−80 °C) until the day of analysis and served for further quantitative estimation kynurenic acid content. Blanks of two types were used. "No tissue" blanks contained all of the incubation buffer components except for the aortic tissue, whereas "no endothelium" blanks contained endothelium-denuded aortic rings. At least 6 wells were used for each studied concentration and the experiments were repeated twice.

2.4. In vivo studies

Animals were given DL-homocysteine (1.3 mmol/kg, i.p.), in the volume of 1 ml/200 g of body weight, and sacrificed 15 or 60 min later. Control animals received solvent i.p. Aortas were harvested as described above, at the appropriate time-points, and the endothelial layer was gently removed by rubbing and rinsing. Collected samples of 1 ml volume (from each animal) were sonicated, acidified (0.1 ml of 1 N HCl and 14 μl of 50% TCA), and centrifuged; supernatant was stored (−80 °C) until the day of analysis. The protein concentration within homogenates was determined using a modified Bradford assay (Bio-Rad). Samples of blood (1 ml) were collected into tubes containing citrate,

centrifuged (13900 g; 4 °C; 10 min), and the supernatant was stored (−80 °C) until the day of further analyses.

2.5. Quantitative analysis of kynurenic acid

The supernatants, obtained as described above, were applied to the columns containing cation-exchange resin (Dowex 50 W^+), prewashed with 0.1 M HCl. Columns were subsequently washed with 1 ml of 0.1 M HCl and 1 ml of water. Kynurenic acid was eluted with 2.5 ml of water. Eluted kynurenic acid was subjected to the HPLC system (Varian, USA) and quantified fluorimetrically (excitation 246 nm, emission 404 nm) using ESA catecholamine HR-80, 3 μm, C_{18} reverse-phase column (Stazka et al., 2002). The mean control production of kynurenic acid in vitro, in the presence of 50 μM L-kynurenine, was 1.85±0.29 pmol/mg of aortic tissue/1 h.

2.6. Statistical analyses

Data are reported as percent of control values±S.D. Statistical analyses were performed with on ay analysis of variance (ANOVA) followed by the adjustment of P value by the Bonferroni method. The concentration of a compound necessary to induce the 50% inhibition of kynurenic acid synthesis (IC_{50}), with 95% confidence limits, was calculated using the computerized linear regression analysis of quantal log dose-probit function.

3. Results

3.1. Effect of DL-homocysteine and S-adenosylhomocysteine on kynurenic acid production in vitro

DL-homocysteine used at the concentrations of 40, 60, 80 and 100 μM increased the synthesis of kynurenic acid to 120.0%, 134.5%, 136.0% and 130.3% of control (all $P<0.05$), respectively. DL-homocysteine at 400 and 500 μM inhibited the formation of kynurenic acid to 82.0% and 61.1% of control (both $P<0.05$), respectively (Fig. 1A). Similarly, S-adenosylhomocysteine at 40, 60 and 80 μM increased kynurenic acid production to 119.6%, 128.0% and 124.0% of control (all $P<0.05$), whereas at 400 and 500 μM diminished kynurenic acid synthesis to 76.3% and 62.0% of control (both $P<0.05$), respectively (Fig. 1B). "No tissue" and "no endothelium" blanks did not produce any measurable amounts of kynurenic acid (data not shown).

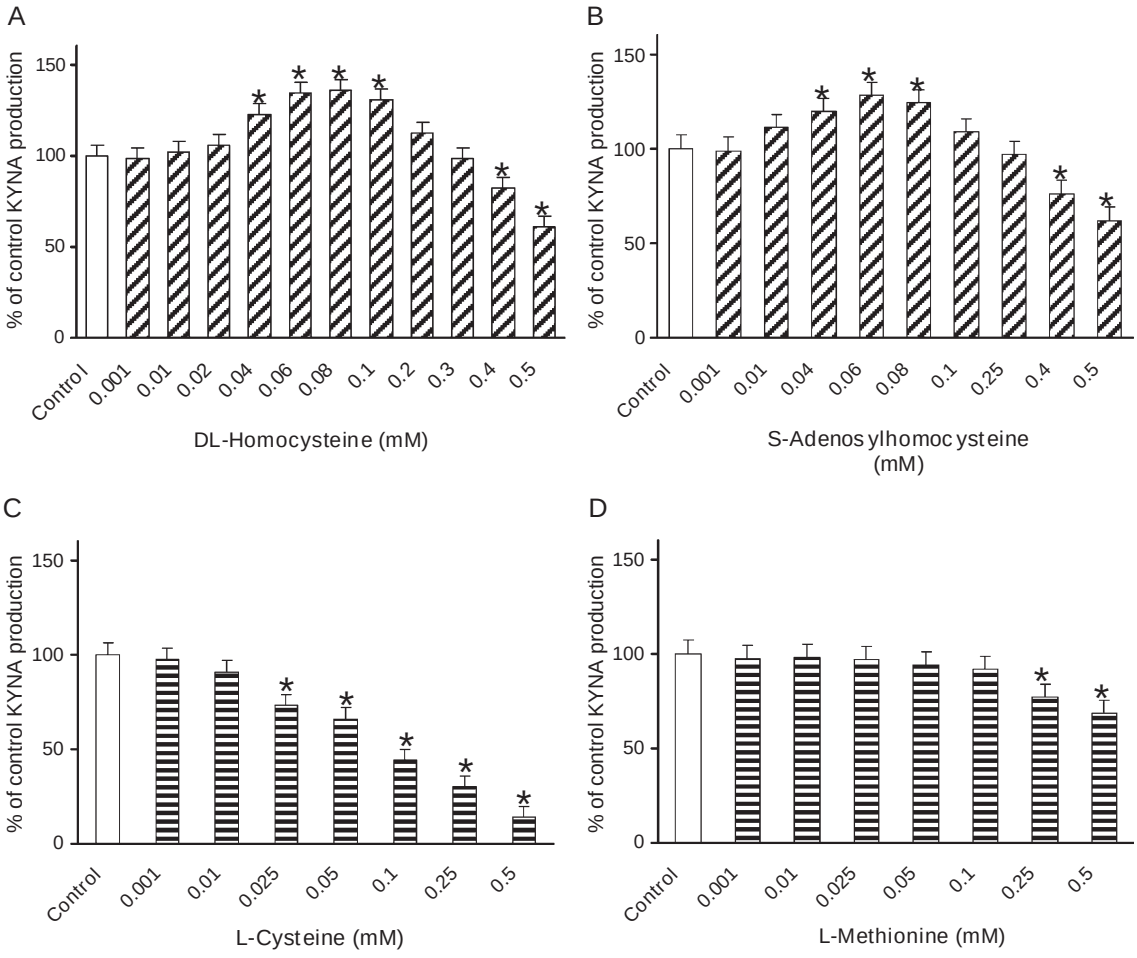

Fig. 1. The effect of DL-homocysteine (A), S-adenosylhomocysteine (B), L-cysteine (C) and L-methionine (D) on the endothelium-dependent kynurenic acid production in rat aortic slices. Data are mean values±S.D. of six determinations vs. control (100%). The statistical comparisons of results were performed using one way ANOVA with post hoc comparisons according to the Bonferroni method ($^aP<0.05$ vs. control).

3.2. Effect of L-cysteine and L-methionine on kynurenic acid production in vitro

To assess the potential involvement of sulfhydryl groups in the effect displayed by DL-homocysteine we have investigated the action of two thiol-containing compounds metabolically related to homocysteine, L-methionine and L-cysteine, on the kynurenic acid production. L-cysteine acted only as a potent inhibitor of kynurenic acid synthesis, reaching the IC_{50} value of 70.6 [48.4–102.9] μM (Fig. 1C). L-methionine at 0.25 and 0.5 mM concentration reduced kynurenic acid production to 77.0% and 68.4% of control (both $P<0.05$), respectively (Fig. 1D). "No tissue" and "no endothelium" blanks did not produce any measurable amounts of kynurenic acid.

3.3. Effect of DL-homocysteine on the level of kynurenic acid in serum and within endothelium

The administration of DL-homocysteine increased serum level of kynurenic acid (studied at 15 and 60 min post injection) from 23.7±7.1 to 60.7±14.2 ($P<0.01$) and to 55.7±13.6 ($P<0.01$) pmol/ml, respectively. The endogenous content of kynurenic acid within aortic endothelium was increased from 51.6±5.8 to 73.2±9.4 fmol/μg of protein (15 min; $P<0.01$) and returned to control value 46.7±8.5 fmol/μg of protein (60 min).

3.4. Effect of L-glycine and CGS 19755 on the DL-homocysteine- and S-adenosylhomocysteine-evoked changes in kynurenic acid synthesis in vitro

In order to evaluate the potential role of NMDA receptor stimulation in the action of DL-homocysteine and *S*-adenosylhomocysteine, the effects of L-glycine and CGS 19755 were studied in vitro. L-glycine (1 mM) and CGS 19755 (1 mM) have not affected the basic production of kynurenic acid (99±6% and 98±5% of control, respectively). Glycine (1 mM) has influenced neither the enhancement nor the inhibition of kynurenic acid synthesis evoked by 0.08 (133±7 vs. 137±5) or 0.5 mM DL-homocysteine (71±6% vs. 64±7% of control, respectively). Similarly, CGS 19755 (1 mM) has affected neither the synthesis of kynurenic acid stimulated by 0.08 mM DL-homocysteine (141±8% vs. 137±7% of control) nor inhibited by 0.5 mM DL-homocysteine (68±6% vs. 64±7% of control).

Glycine (1 mM) has not changed the *S*-adenosylhomocysteine-induced stimulation (0.06 mM) or inhibition (0.75 mM) of kynurenic acid production (132±8% vs. 127±6% and 67±4% vs. 63±8% of control, respectively). Similarly, CGS 19755 (1 mM) has not influenced kynurenic acid synthesis stimulated by 0.06 mM *S*-adenosylhomocysteine (129±7% vs. 137±7% of control) or inhibited by 0.75 mM *S*-adenosylhomocysteine (65±7% vs. 64±7% of control). "No tissue" and "no endothelium" blanks did not produce any measurable amounts of kynurenic acid.

3.5. The influence of L-phenylalanine and L-leucine on the stimulation of kynurenic acid synthesis exerted by S-adenosylhomocysteine

L-leucine and L-phenylalanine at 0.2 mM concentration reduced the basal kynurenic acid production in aortic rings to 59.2% ($P<0.001$) and 47.5% ($P<0.001$) of control, respectively (Fig. 2). L-leucine and L-phenylalanine (both 0.2 mM) prevented the *S*-adenosylhomocysteine-induced increase of kynurenic acid production (52.0% vs. 132.1% and 55.4% vs. 150.0% of control, respectively) (Fig. 2).

4. Discussion

In the present study, treatment of aortic rings with DL-homocysteine induced significant, biphasic changes of endothelium-dependent production of kynurenic acid. DL-homocysteine at 40–100 μM concentrations, relevant to values typical for hyperhomocysteinemia, increased the formation of kynurenic acid, whereas at concentrations ≥400 mM reduced its synthesis. Similar, biphasic effect was also elicited by *S*-adenosylhomocysteine. In contrast, two other thiol-containing compounds, L-methionine and L-cysteine, acted only as the inhibitors of kynurenic acid production. In vivo, application of DL-homocysteine

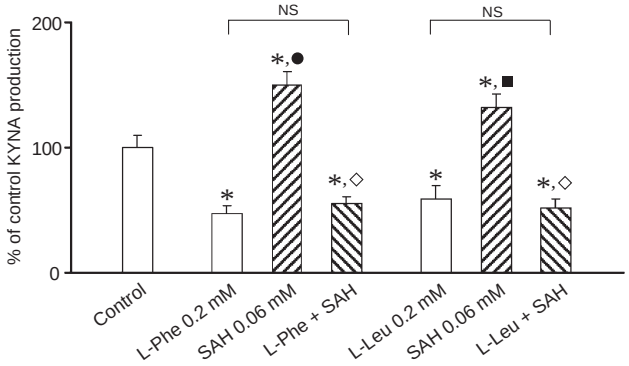

Fig. 2. The influence of L-phenylalanine (L-Phe) and L-leucine (L-Leu) on the *S*-adenosylhomocysteine (SAH)-evoked stimulation of kynurenic acid synthesis in rat aortic rings. Data are mean values±S.D. of six determinations vs. control (100%). The statistical comparisons of results were performed using one way ANOVA with post hoc comparisons according to the Bonferroni method (*$P<0.05$ vs. control; ●$P<0.05$ vs. L-phenylalanine; ■$P<0.05$ vs. L-leucine; ◇$P<0.05$ vs. *S*-adenosylhomocysteine).

increased the content of kynurenic acid within endothelium and raised kynurenic acid serum level.

In the brain, kynurenic acid interacts preferentially with NMDA glutamatergic receptors (at micromolar concentrations) and α7-nicotinic cholinergic receptors (at nanomolar levels) (Hilmas et al., 2001; Stone, 2001). Despite controversies, it is believed that kynurenic acid may locally attain the concentrations high enough to modulate not only the activation of á7-nicotinic cholinergic receptors but also of NMDA receptors (Hilmas et al., 2001; Stone, 2001; Alkondon et al., 2004). Glutamatergic as well as nicotinic receptors are expressed in the cardiovascular system, including endothelium (Wang et al., 2001; Zhu and Liu, 2004). Peripheral, non-neuronal nicotinic receptors appear to be involved in the regulation of cellular metabolism, including mitosis or trophic functions (Wessler et al., 1998). The role played by kynurenic acid outside CNS remains to be established. However, in the view of available data, it cannot be excluded that kynurenic acid might modulate the function of endothelium. In fact, the serum level of kynurenic acid falls within the nanomolar range, thus sufficient to modify the activity of cholinergic nicotinic receptors.

It is considered that 5% to 7% of the general population and up to 40% of patients with coronary heart disease have mild to moderate hyperhomocysteinemia, with homocysteine levels ranging between 15 and 50 μM (Durand et al., 2001; Medina et al., 2001). Severe congenital homocystinuria may result in plasma homocysteine level of 200–400 μM (Durand et al., 2001; Medina et al., 2001). The concentrations of DL-homocysteine effective here, in vitro, are comparable with values found in humans manifesting mild hyperhomocysteinemia. Thus, the present results seem to be potentially relevant to clinical situation.

Prior work revealed the ability of rat aortic endothelium to produce kynurenic acid de novo and to release it subsequently into the extracellular compartment (Stazka et al., 2002). As shown here, rat aortic endothelium contains measurable amounts of endogenous kynurenic acid. Our original observation was very recently confirmed by others demonstrating that bovine endothelial cells produce kynurenic acid de novo (Wejksza et al., 2004). The authors have reported that DL-homocysteine may reduce the production of kynurenic acid, but they have not noticed the stimulatory action of DL-homocysteine (Wejksza et al., 2004), most probably because it occurs over a relatively narrow range of low concentrations.

The biphasic activity of DL-homocysteine has been noted previously. DL-homocysteine, but not L-cysteine, augmented at low concentrations and inhibited at high concentrations the activity of latent elastolytic metalloproteinase proMMP-2, an enzyme remodelling the extracellular matrix in arterial walls (Bescond et al., 1999). DL-homocysteine and *S*-adenosylhomocysteine were also shown to biphasically modulate the brain production of kynurenic acid under in vitro and in vivo conditions (Luchowska et al., 2005).

These data suggest that DL-homocysteine might enhance kynurenic acid production either directly or indirectly, following conversion to *S*-adenosylhomocysteine. In fact, the increased tissue levels of *S*-adenosylhomocysteine are associated with the endothelial dysfunction during hyperhomocysteinemia (Dayal et al., 2001; Wang et al., 1997). Moreover, the serum levels of *S*-adenosylhomocysteine are increased when DL-homocysteine concentrations are of 10–50 μM (Wang et al., 1997), i.e. similar to these effective here. The precise mechanism underlying the activity of DL-homocysteine and *S*-adenosylhomocysteine is unclear. However, it is known that *S*-adenosylhomocysteine may enhance the uptake of certain monoamines in vitro and in vivo (Fonlupt et al., 1979). Therefore, we hypothesized that the endothelial uptake of kynurenic acid bioprecursor, L-kynurenine, can also be augmented by *S*-adenosylhomocysteine, as was the case in cortical slices (Luchowska et al., 2005). In aortic rings, L-kynurenine uptake inhibitors, L-leucine or L-phenylalanine, prevented the *S*-adenosylhomocysteine-evoked stimulation of kynurenic acid synthesis. Thus, the endothelial action of *S*-adenosylhomocysteine seems to depend, at least partially, on the increased uptake of L-kynurenine.

The observed enhancement of endothelial kynurenic acid synthesis elicited by DL-homocysteine might also result from the increased activity of kynurenic acid biosynthetic enzyme(s). Presence of kynurenine aminotransferase within endothelial bovine cells was recently demonstrated immunohistochemically (Wejksza et al., 2004), but this endothelial enzyme(s) has not been biochemically characterized so far. Kynurenine aminotransferases isolated from brain and peripheral organs vary as regards e.g. their pH optimum, cofactor requirements, and other features (Baran et al., 1997; Buchli et al., 1995; Noguchi et al., 1975). Therefore, the activity of endothelial enzyme(s) cannot be assessed prior to the basic biochemical characterization. Noteworthy, among studied here substances, none was able to enhance the activity of brain kynurenine aminotransferases. At high concentrations though, all of the compounds inhibited either the activity of kynurenine aminotransferase I or II (Luchowska et al., 2005). Therefore, a direct, DL-homocysteine-evoked, stimulation of kynurenic acid biosynthetic enzymes seems unlikely. In contrast, the inhibition of kynurenic acid production displayed by DL-homocysteine, *S*-adenosylhomocysteine, L-cysteine and L-methionine might be due to the diminished activity of endothelial biosynthetic enzyme(s).

L-cysteine and L-methionine, as opposed to DL-homocysteine and *S*-adenosylhomocysteine, acted only as the inhibitors of kynurenic acid synthesis in aortic rings. Therefore, similarly as in the cortex (Luchowska et al., 2005), the nonspecific reactivity of sulfhydryl groups does not seem to contribute to the effects induced by DL-homocysteine and *S*-adenosylhomocysteine. L-cysteine is reaching high levels during hyperhomocysteinemia, and its concentration exceeding 275 μM is considered a cardiovas-

cular disease risk factor (El-Khairy et al., 2001). In aortic rings, L-cysteine potently diminished kynurenic acid production, in contrast to its weak action in the brain (Luchowska et al., 2005). L-cysteine was effective in a low, micromolar range, comparable with values found in human (approx. 250 μM) and rat serum (approx. 150 μM) (El-Khairy et al., 2001; Richie et al., 2004). L-methionine also inhibited the endothelial kynurenic acid synthesis, but it acted at concentrations by approx. 10 fold higher than these occurring in human serum (Suliman et al., 1997).

In the brain, homocysteine interacts with ionotropic glutamate receptors of *N*-methyl-D-aspartate (NMDA) type and with metabotropic glutamate receptors (Lipton et al., 1997; Lazarewicz et al., 2003). The presence of glutamate ionotropic receptors in the cardiovascular system, including endothelium, has been postulated (Crespi et al., 2000; Gill et al., 1998; Zhu and Liu, 2004). However, neither glycine, a co-agonist of strychnine-insensitive glycine site within NMDA receptor, nor CGS 19755, a competitive antagonist of glutamate site, were able to influence either the basal or DL-homocysteine-and *S*-adenosylhomocysteine-altered production of kynurenic acid. Therefore, the involvement of NMDA receptors in the regulation of endothelial kynurenic acid production seems doubtful.

Impaired brain synthesis of kynurenic acid was implicated in the development of neurodegeneration and seizures (Jauch et al., 1995; Urbanska et al., 1991), whereas an increase of kynurenic acid formation may play a role in affective disorders or impaired cognition (Baran et al., 1999; Schwarcz et al., 2001). The function of kynurenic acid in the cardiovascular system is not yet recognized. Preliminary clinical studies revealed that there is a positive correlation between homocysteine and kynurenic acid levels in the serum. Moreover, serum kynurenic acid level tends to be increased among the patients with ischemic heart disease (Urbanska et al., submitted for publication).

In resume, the observed effects of DL-homocysteine seem to be exerted, at least in part, via *S*-adenosylhomocysteine, which probably stimulates the uptake of kynurenic acid bioprecursor, L-kynurenine. At higher concentrations of DL-homocysteine, the inhibition of kynurenic acid biosynthetic enzyme(s) most likely prevails, and the compound, directly or indirectly, diminishes the endothelial synthesis of kynurenic acid. The potential role of endothelium-derived kynurenic acid in the cardiovascular physiology and pathology requires further studies.

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