From Genome Wide Association Study to arterial wall protection: homoarginine effect on intimal hyperplasia in balloon-injured rat carotids

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Running title: Homoarginine reduces carotid intimal hyperplasia

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Dear Sirs,

Despite highly effective measures to control conventional risk factors, many cardiovascular events still occur. For this reason, identifying new players in cardiovascular health is crucial to improve risk prediction and to identify novel targets of treatment (1). Recent studies have indicated that homoarginine metabolism may be particularly relevant to cardiovascular health (2-4). Homoarginine is an arginine homologue whose physiological role is unknown, that differs from arginine by an additional methylene group. Because of this structural similarity, it has been suggested that homoarginine may be a substrate, alternative to arginine, for nitric oxide synthase (5). Additionally, it may also indirectly increase nitric oxide (NO) production by inhibiting arginase activity, thus raising arginine availability (6). In support of these evidences, homoarginine levels have been associated with endothelial function (3, 7). Data from clinical studies have indicated that low homoarginine concentrations independently predict mortality from cardiovascular disease, including sudden cardiac death, heart failure, and fatal ischemic stroke (4, 8-11). Two independent Genome-Wide Association Studies (GWAS) documented a strong association between serum homoarginine concentration and the region on chromosome 15 containing the arginine:glycine amidinotransferase (AGAT) gene (12, 13). AGAT plays a central role in energy metabolism by catalyzing the conversion of arginine and glycine to ornithine and guanidinoacetate, which is subsequently methylated to creatine. However, when AGAT uses lysine instead of glycine, homoarginine is formed (14). Interestingly, Choe et al have shown that AGAT knockout mice are characterized by extremely low levels of homoarginine and that, when experimental ischemic stroke was induced, these mice had larger infarct volumes and worse neurological deficits compared to wild-type animals. Importantly, these features were attenuated by homoarginine supplementation (12).

The possible role of homoarginine on smooth muscle cell proliferation and migration that can occur after arterial balloon angioplasty has never been explored. To this aim, balloon injury was performed in the left carotid artery of Sprague-Dawley rats, followed by the insertion of a cannula into the right jugular vein for continuous i.v. drug administration, as previously described (15). Thirty-six male Sprague-Dawley rats were used for the study and randomly divided into three treatment groups: group 1, infused with saline (CTR); group 2, infused with L-arginine (30 mg/kg/day in saline, ARG); group 3, infused with L-homoarginine (30 mg/kg/day in saline, HOMO). The intravenous continuous infusion lasted 14 days, starting from the day of the arterial injury, and was achieved by connecting the cannula inserted into the right jugular vein to an osmotic infusion pump containing 2 ml of the solutions described above. Systolic blood pressure was measured by tail cuff volume-oscillometric method in conscious animals, before and 14 days after balloon injury. At the end of drug treatments, blood was collected, rats were humanly sacrificed and left carotids were harvested for histological analyses (see Suppl. Methods, available on-line at www.thrombosis-online.com).

No signs of toxicity by treatments were detected. Homoarginine-treated rats showed a significant reduction of the vessel intimal/medial area ratio compared to that of controls ($p<0.05$; Figure 1). This inhibition of neointimal hyperplasia was similar to that observed in the ARG group, where the intimal/medial ratio was also lower than that measured in control rats, supporting previous results (15) ($p<0.05$ vs controls; Figure 1). No differences were observed in the medial area among the three experimental groups (data not shown). No differences were also detected in systolic blood pressure among the rats at baseline (data not shown), as well as at the end of the treatment (156±15 mmHg, 159±11 mmHg, and 143±14 mmHg in CTR, ARG, and HOMO groups, respectively, $p>0.05$).

Homoarginine, arginine and ornithine concentrations were measured in blood collected from fasted rats at the end of the treatments. Homoarginine serum levels were dramatically high in

homoarginine-treated rats compared to both CTR and ARG groups (38.5±8.4 μM, 1.2±0.1 μM, and 1.1±0.3 μM in HOMO, ARG, and CTR groups, respectively; $p<0.0001$). Plasma arginine concentration was instead significantly increased in both ARG and HOMO groups compared to controls (137.3±15.6 μM, 139.3±25.9 μM, and 116.2±12.9 μM in ARG, HOMO and CTR groups, respectively; $p<0.05$). The observed increase of arginine by homoarginine treatment supports previous observations suggesting that homoarginine may interfere with arginine metabolism by inhibiting arginase activity (6).

To evaluate if homoarginine and arginine treatments could result in higher NO availability, nitrite serum concentrations were measured as index of intracellular NO production and endothelial NO synthase activity (16). Indeed, ARG and HOMO groups had higher levels of nitrite compared to saline-treated rats (2.4±1.2 μM, 2.2±0.8 μM, and 1.1±0.4 μM in L-ARG, HOMO and CTR groups, respectively; $p<0.05$). These results support the hypothesis that homoarginine may exert its antiproliferative effect by increasing NO release from vascular cells (17).

Finally, in the HOMO group a significant increase in the serum concentration of ornithine was observed compared to CTR and ARG groups (91.0±12.9 μM, 73.8±11.7 μM, and 69.4±9.2 μM in HOMO, ARG and CTR groups, respectively; $p<0.05$). The observed increase of ornithine levels may contribute to the antiproliferative effect observed in homoarginine-treated rats, since ornithine has been shown to increase NO avaibility, acting as arginase inhibitor (6, 17).

In summary, the present study shows, for the first time, that an *in vivo* administration of homoarginine is able to inhibit neointimal formation, at least in part, by increasing both arginine availability and NO production. Taken together these results corroborate previous clinical evidences showing an association between homoarginine levels, endothelial function and cardiovascular health (3, 4, 10, 11).

Conflict of interest

None declared.

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Figure legends

Figure 1. Homoarginine treatment inhibited neointimal hyperplasia in balloon-injured rat carotids. Top and bottom-left: Representative photomicrographs of cross-sections of the left carotid artery harvested from saline- (CTR), arginine- (ARG) and homoarginine- (HOMO) treated rats. **Bottom-right:** Ratio of intimal to medial areas measured in CTR, ARG and HOMO groups 14 days after balloon injury.

Data are expressed as: mean ± SD (n=12). *$p<0.05$ vs CTR. Bar in top-left panel = 100 μ.

Figure 1