Age-related changes in the protective effect of chronic administration of L-arginine on post-ischemic recovery of endothelial function

Koki Nakamura\textsuperscript{a}, Sharif Al-Ruzzeh\textsuperscript{a}, Adrian H. Chester\textsuperscript{a}, Ann Dewar\textsuperscript{b}, Steve Rothery\textsuperscript{c}, Nicholas J. Severs\textsuperscript{c}, Magdi H. Yacoub\textsuperscript{a}, Mohamed Amrani\textsuperscript{a,1,*}

\textsuperscript{a}National Heart and Lung Institute, Heart Science Centre, Harefield Hospital, Harefield, Middlesex UB9 6JH, UK
\textsuperscript{b}Electron Microscopy Unit, Brompton Hospital, London, UK
\textsuperscript{c}Cell Biology Department, Brompton Hospital, London, UK

Received 6 August 2002; received in revised form 23 December 2002; accepted 7 January 2003

Abstract

Objective: The aim of this study was to assess the effect of chronic administration of L-arginine (LA) on vascular functions as well as its age-related changes.

Methods: Male Sprague–Dawley rats aged 1, 4, 8 and 16 months were divided into control and LA groups, which were administered LA (4 mg/ml) for 6 weeks. Isolated heart perfusion was performed, followed by cardioplegic arrest for 4 h at 4°C and reperfusion. Vascular functions were assessed through observations of pre-/post-ischemic coronary flow response to 5-hydroxytryptamine (5-HT) and glyceryl trinitrate (GTN). Ultrastructure was studied after the same ischemia-reperfusion.

Results: A significant improvement of percentage recovery (post-/pre-ischemic value) of response to 5-HT were seen in 4 and 8 months LA group when compared to the control (84.2 ± 14.0 vs. 33.9 ± 12.5 (P, 0.05) and 97.0 ± 23.2 vs. 21.5 ± 9.7 (P < 0.05), respectively). Furthermore, 8 months LA group had better percentage recovery of response to GTN (124.5 ± 41.6 vs. 47.7 ± 6.3, P, 0.05). Ultrastructural study showed no significant differences between the groups in any age.

Conclusions: Chronic oral administration of LA enhanced the post-ischemic recovery of vascular function in the young adult and adult hearts, but not in the infant and elderly.

Keywords: Aging; Endothelial function; Ischemia; Nitric oxide; Reperfusion

1. Introduction

Coronary endothelial dysfunction is a common feature of ischemia-reperfusion injury. Many causes have been described including impairment of the endothelial production of nitric oxide (NO) [1–3]. NO has numerous beneficial effects which include vasodilatory properties, an inhibitory action on platelet aggregation and antioxidative effects [4]. Endothelial dysfunction, especially impaired endothelium-dependent vasodilatation, is a common characteristic of aging [5–9]. Age-related reductions in endothelium-dependent vasodilatation have been observed in human coronary artery [5]. Furthermore, basal and stimulated release of NO by coronary endothelium deteriorates with age [6].

Acute administration of L-arginine (LA) has been widely used as a protective strategy against endothelial dysfunction. It was demonstrated that exogenous LA improved the post-ischemic recovery of cardiac mechanical function and coronary flow (CF) after cardioplegic arrest and ischemia by stimulation of NO production when given in the reperfusate [2,3] or during ischemia [10]. In contrast, some studies suggested that LA could be detrimental to the heart function when given before or after ischemia [11,12].

The effect of chronic administration of LA is still controversial. Whilst some reports showed the beneficial effects of chronic oral LA supplementation on attenuating hypertrophy [13], improving heart failure [14], reducing intimal hyperplasia [15] and preserving isolated aortic ring relaxation and cardiac functions [16], other investigations showed no beneficial effects on endothelium-dependent vasodilatation and inflammation markers [17] and left ventricular function [18].

The role of chronic administration of LA on vascular
function in a clinically relevant surgical model has not been clearly defined. The aim of the present study was to evaluate effects of chronic oral LA administration on post-ischemic vascular recovery following a prolonged cardioplegic arrest as well as its age-related changes.

2. Methods

2.1. Animals and grouping

Male Sprague–Dawley rats aged 1, 4, 8 and 16 months were used. Each age group represented infant, young adult, adult and elderly, respectively. In all studies, animals received humane care in compliance with the ‘Principles of Laboratory Animal Care’ formulated by the National Society for Medical Research and the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the Institute of Laboratory Animal Resources and published by the national Institutes of Health (NIH Publication No. 85-23, revised 1996). LA (4 mg/ml; Sigma, Poole, Dorset, UK) was administered in the drinking water of the rats in the LA group for 6 weeks, while the control rats had been kept with normal drinking water for the same period. Daily water intake was recorded. For the vascular function study, control group included 11, nine, seven and eight animals aged 1, 4, 8 and 16 months, respectively, while LA group included 11, seven, eight and eight. For the morphological study, four animals were used in each group.

2.2. Experimental preparation and time course

The isolated rat heart preparation used in this study has already been described in detail elsewhere [19]. Krebs–Henseleit bicarbonate buffer [2] was gassed with 95% O₂ and 5% CO₂ at 37°C and perfusion pressure was continuously maintained by keeping reservoirs 100 cm above the heart through the experiment. Ischemic cardiac arrest was produced by clamping the aortic cannula. At this time, the hearts were subjected to a 10 ml hypothermic (4°C) coronary infusion with St. Thomas’s Hospital No.1 solution and then kept immersed in the same solution for 4 h at 4°C maintained by cooling circuits. Time course is shown in Fig. 1: vascular function study in Fig. 1a and morphological study in Fig. 1b. Blood samples were collected when the hearts were excised. One whole blood sample (0.5 ml) was mixed with 0.5 ml of 4% PCA and was frozen in liquid nitrogen until LA assay. Another sample was centrifuged, then plasma was isolated and stored at −70°C until NO assay.

2.3. Chemicals

St. Thomas’s Hospital cardioplegic solution No. 1, supplied as concentrate (David Bull Laboratories, Mulgrave, Victoria, Australia), was diluted in Ringer’s solution (Travenol Laboratories, Thetford, Norfolk, UK) and passed through a 0.2 μm filter (Pall Biomedical, Glen Cove, NY). Glyceryl trinitrate (GTN; David Bull) and 5-hydroxytryptamine (5-HT; Sigma Chemical Co, Poole, Dorset, UK) were diluted in Krebs solution.

2.4. Endothelial and vascular smooth muscle function studies

Endothelial and vascular smooth muscle functions were assessed through observations of pre-ischemic and post-ischemic coronary flow responses to 5-HT (10⁻⁵ mol/l) and GTN (15 mg/l), respectively [1]. Langendorff perfusion was initiated for 20 min to allow steady coronary flow to be reached. Then the infusion was switched to one containing 5-HT and then washed out with Krebs buffer. This was followed by perfusion with GTN and washout period with Krebs buffer. CF was monitored by an electromagnetic flow probe (20 ml ECM2; Scalar, Delft, Holland), which was connected to a flowmeter (MDL 1401; Scalar). After the ischemic period, the hearts were subjected to the same sequence of perfusion including the vascular function studies. During perfusion with any substance, steady CF was allowed to be reached before further perfusion was initiated.

CF was expressed in ml/min. The vasodilatory responses to 5-HT and GTN were expressed as a percentage of change in the baseline CF. Post-ischemic recovery of response to 5-HT and GTN was expressed as a percentage of individual pre-ischemic control response. The post-ischemic recovery of CF was expressed as the percentage of the pre-ischemic value.

2.5. Endothelial and vascular smooth muscle ultrastructure studies

In a parallel series of experiments, vascular morphology was assessed (n = 4 in each group). Langendorff perfusion was initiated at 37°C for 20 min and the heart was subjected to the cardioplegic arrest for 4 h at 4°C followed by reperfusion at 37°C for 20 min. Then the heart was perfusion-fixed with 2.5% glutaraldehyde in Krebs buffer for 5 min. The left ventricular free wall was dissected and sliced transversely to include epicardial and endocardial surfaces, then the tissues were further fixed for 2 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The tissue was post-fixed and embedded in Araldite CY212 as previously described [1]. Semi-thin (0.5-1 μm) sections were stained with 1% toluidine blue in 1% borax-50% ethanol, and examined with a light microscope. Two separate blocks in each heart were examined, consequently, eight different sections in each group were analyzed.

Endothelial and vascular smooth muscle morphology was microscopically classified as undamaged (U), mildly damaged (Mi), moderately damaged (Mo), or severely damaged (S) [1]. Undamaged vessels had an intact
endothelium lining the entire intimal surface with no damage. Smooth muscle was invariably well preserved in these vessels. Mildly damaged vessels were defined by the presence of an intact endothelium over \( \geq 90\% \) of the luminal surface in which focal lifting of the endothelium or minor vacuolation was apparent. Smooth muscle was well preserved or showed only minor vacuolation. Moderately damaged vessels were defined by the loss of major portions ( \( \leq 50\% \) ) of the endothelium, and vacuolation and lifting were common. Underlying smooth muscle was frequently vacuolated. Severely damaged vessels were defined by the absence of endothelium over 50\% of their surface with extensive structural damage of remaining cells. Smooth muscle was usually severely damaged in these vessels.

2.6. Measurement of LA and NOx level

LA level was analyzed using a modified enzymatic method [20]. Blood samples were homogenized and microfuged followed by taking supernatant. After neutralization, the supernatant was analyzed by a spectrophotometer using an enzymatic reaction catalyzed by octopine dehydrogenase: pyruvate + L-arginine + NADH \( \Leftrightarrow \) octopine + H\(_2\)O + NAD. The change of absorbance at 340 nm was monitored and LA level was calculated. To determine total NO production, the amount of its breakdown product (nitrite) was assayed with a chemiluminescence method using NO analyzer (Sievers 270, Colo) [2]. Nitrite is measured as an index of total NO production, as NO\(_2\) is the principal oxidation product in an aqueous solution devoid of any biologic contaminants.

2.7. Statistical analysis

Data were analyzed using unpaired Student’s \( t \)-test if normal distribution was confirmed by \( F \)-test, otherwise, Mann Whitney’s \( U \)-test was applied for comparisons between the LA and control groups. Correlation between the plasma levels of LA and the endothelial function data was also statistically examined. For the morphological analysis, two factor factorial analysis of variance (ANOVA) was used. Significance was assumed when \( P \)-value was less than 0.05. Values were given as mean \( \pm \) standard error of the mean (SEM).

3. Results

3.1. Levels of LA and NOx in plasma

Mean water intake in the LA group was 32.9 \( \pm \) 2.4, 40.4 \( \pm \) 3.1, 30.9 \( \pm \) 10.6 and 31.9 \( \pm \) 5.3 (ml/day) in 1, 4, 8 and 16 months animals, respectively. Consequently, LA supplementation was 131.6 \( \pm \) 9.6, 161.6 \( \pm \) 12.4, 123.6 \( \pm \) 42.4 and 127.6 \( \pm \) 21.2 (mg/day) in 1, 4, 8 and 16 months animals, respectively. In total, plasma LA level was significantly greater in the LA group compared with the control (288.9 \( \pm \) 12.9 vs. 227.6 \( \pm \) 14.2 \( \mu \)mol/l, \( P = 0.0019 \)). The value in the each age group is shown in Table 1. As shown in Table 2, there were no significant differences of plasma NO level (basal level of NO) between the two groups (20.2 \( \pm \) 1.9 \( \mu \)mol/l in LA vs. 19.9 \( \pm \) 1.2 \( \mu \)mol/l in control, NS).
3.2. Preservation of endothelial and vascular smooth muscle functions

There was no significant difference of percentage recovery of CF (post-/pre-ischemic value × 100) between LA and control groups. On the other hand, significantly better percentage recovery response to 5-HT (post-/pre-ischemic value × 100) was seen in 4 and 8 months of LA group compared with control (84.2 ± 14.0% vs. 33.9 ± 12.5% and 97.0 ± 23.2% vs. 21.5 ± 9.7%, respectively; \( P < 0.05 \)), whereas there were no significant differences between the two groups in 1 and 16 months (39.9 ± 12.4% vs. 27.4 ± 22.2% and 14.1 ± 31.6% vs. 23.9 ± 35.7%, respectively; NS). Furthermore, percentage recovery of response to GTN (post-/pre-ischemic value × 100) was significantly higher in 8 months of LA group than in control (124.5 ± 41.6% vs. 47.7 ± 6.3%, \( P < 0.05 \)) although no significant differences were seen in other age groups. These results are compared graphically in Figs. 2–4. There was no significant correlation between the plasma levels of LA and the endothelial function data.

3.3. Preservation of endothelial and vascular smooth muscle morphology

In all specimens, capillary and myocyte structures remained well preserved with small variations. Because clear-cut and consistent structural differences were not obvious by qualitative comparison of the groups, a semiquantitative comparison, as described previously, were made. However, no significant differences were detected between LA and control groups in any age animals. In control group (average of all age animals), undamaged (U) was 70.0 ± 6.6%, mildly damaged (Mi) 26.8 ± 5.3%, moderately damaged (Mo) 2.8 ± 1.8% and severely damaged (S) 0.4 ± 0.4%. Similarly, in LA group, U was 60.2 ± 8.6%, Mi 30.1 ± 6.0%, Mo 9.4 ± 4.8% and S 0.3 ± 0.3% (NS). Results in each group are shown graphically in Fig. 5.

4. Discussion

The present study showed that chronic administration of LA significantly improved the coronary endothelial-dependent vasodilatation reflecting the stimulated release of NO in young adult (4 months) and adult (8 months) groups. No

Table 1

<table>
<thead>
<tr>
<th>Age</th>
<th>LA (μmol/l)</th>
<th>Control (μmol/l)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>271.6 ± 24.8</td>
<td>205.4 ± 31.0</td>
<td>0.10</td>
</tr>
<tr>
<td>4 months</td>
<td>289.3 ± 26.1</td>
<td>213.1 ± 28.7</td>
<td>0.06</td>
</tr>
<tr>
<td>8 months</td>
<td>298.2 ± 22.8</td>
<td>255.4 ± 14.6</td>
<td>0.16</td>
</tr>
<tr>
<td>16 months</td>
<td>328.8 ± 20.1</td>
<td>257.5 ± 25.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Total</td>
<td>288.9 ± 12.9</td>
<td>227.6 ± 14.2</td>
<td>0.0019</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Age</th>
<th>LA (μmol/l)</th>
<th>Control (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>22.9 ± 4.2</td>
<td>19.5 ± 2.7</td>
</tr>
<tr>
<td>4 months</td>
<td>21.5 ± 5.4</td>
<td>17.9 ± 1.5</td>
</tr>
<tr>
<td>8 months</td>
<td>19.4 ± 2.6</td>
<td>20.6 ± 2.4</td>
</tr>
<tr>
<td>16 months</td>
<td>15.8 ± 0.9</td>
<td>21.9 ± 2.8</td>
</tr>
<tr>
<td>Total</td>
<td>20.2 ± 1.9</td>
<td>19.9 ± 1.2</td>
</tr>
</tbody>
</table>

Fig. 2. Baseline coronary flow recovery. Pre-and post-ischemic coronary flow were expressed as ml/min and the value of recovery was calculated as percentage of post-/pre-ischemic value. Closed columns represent LA group and open columns represent control. Each bar represents the mean ± SEM (%). There were no significant differences between the two groups in any age animals.

Fig. 3. Percentage recovery of response to 5-HT. Percent change of coronary flow after applying 5-HT was calculated as percentage response to 5-HT and the percentage recovery was calculated as percentage of post-/pre-ischemic value. Closed columns represent LA group and open columns represent control. Each bar represents the mean ± SEM (%). LA group had a significantly better recovery in 4 and 8 months animals (\(* P < 0.05\)).
improvement was observed in the infant (1 month) and elderly (16 months) group. Ultrastructure studies showed no differences in any groups.

Acute administration of LA is a well established experimental therapeutic modality against post-ischemic endothelial dysfunction, coronary reflow and impaired NO release from the coronary endothelial cells which are well recognized feature of ischemia-reperfusion [2,10]. LA is also known to ameliorate the endothelial dysfunction caused by hypercholesterolemia [21], atherosclerosis [22], and aging [8]. Other beneficial effect of LA include enhanced release of growth hormone, glucagon and insulin, resulting in an improved glucose metabolism [7].

Little is known about the chronic effect of LA upon the cardiac surgical setting although many beneficial effects on the cardiovascular system have been described including an improvement of chronic pulmonary hypertension and pulmonary vascular remodeling [23], attenuation of cardiac hypertrophy [13], amelioration of heart failure [14], reduction in intimal hyperplasia in balloon-injured rat carotid arteries [15]. Our findings suggested that chronic oral administration of LA improved endothelial function reflecting the stimulated release of NO in young adult and adult animals in a cardiac surgical setting. This field of potential clinical application of LA seems to be essentially important because reduction in NO secretion could contribute to both early and late graft failure by exacerbating accelerated coronary artery disease or by reducing CF [2].

Our findings raised three issues including the protective mechanisms, the lack of effect in the infant and elderly groups and the absence of morphological differences in the presence of functional effect of LA.

The protective mechanisms of LA can not be ascertain from our data. Although LA group had a higher plasma LA concentration, there were no significant differences of plasma NOx level between LA and control groups in any age animals. However, the post-ischemic vascular function was better preserved in young adult and adult but not in the infant and elderly animals by LA supplementation. One possibility was that bioavailability of LA in immature and elderly hearts might be relatively lower: for example, storage capacity and transport system of LA might be relatively poor and could be enhanced by exogenous LA.

Fig. 4. Percentage recovery of response to GTN. Percent change of coronary flow after applying GTN was calculated as percentage response to GTN and the percentage recovery was calculated as percentage of post-/pre-ischemic value. Closed columns represent LA group and open columns represent control. Each bar represents the mean ± SEM (%). A significantly better recovery was seen in 8 months of LA group (*P < 0.05).

Fig. 5. Comparison of the post-ischemic endothelial and vascular smooth muscle morphology. Each part of the pie-charts depicts the percentage of sectional views in each category of damage (U = undamaged, Mi = mildly damaged, Mo = moderately damaged, S = severely damaged) for the four hearts in the group. There were no statistical differences between LA and control groups in any age animals.
supplementation. However, from the point of view of LA levels in plasma, it was raised equally in all LA groups so the difference in age groups may relate to inability to utilize the LA effectively in the infant and the elderly.

As for the second question, many reports have shown that the age-related endothelial dysfunction is extremely important to know the mechanism of cardiovascular diseases and events [5,9]. Advancing age is associated with a progressive impairment of endothelium-dependent vasodilatation in the human coronary artery [5]. Using the isolated rat heart perfusion model, we previously demonstrated that basal and stimulated release of NO by coronary endothelium deteriorated with age [6]. Furthermore, Taddei et al. [9] reported that LA potentiated the response to acetylcholine in <30 and 31–45 years old patients, whereas, LA was no longer effective in the patients aged 46 to 60 and >60 years old. Because of the above reasons, we supposed 16 months might be so elderly that LA could not work effectively in our study. On the other hand, resistance to ischemic injury in immature hearts compared with adult hearts has been still controversial. Riva et al. [24] reported that in rats, age-dependent changes in resistance to ischemia presented a biphasic pattern with increasing tolerance up to 23 days of age, followed by a decline. In addition, Chiu et al. [25] described that the immature heart has greater capacity for anaerobic glycolysis and thus, during global ischemia, produces more high-energy phosphates, but is also subject to more rapid accumulation of tissue lactate leading to acidosis and myocardial damage. Regarding to our results, although a certain answer is still unknown, we speculate that 1 month might be too immature for LA to act virtually and in such animals efficiency of LA–NO pathway could be different.

With respect to the last issue, in this study, despite LA group had better recovery of endothelial function, there were no significant differences of endothelial and vascular smooth muscle cell morphology. Possible cause is that ischemia-reperfusion usually injures endothelium and vascular smooth muscle cells functionally at first, then morphologically. It means that the endothelial function study should be more sensitive than morphological study to detect the early stage of injury. In fact, the morphological study showed the endothelial and vascular smooth muscle cells were generally well preserved, furthermore, there were very few ‘severely damaged’ vessels. Therefore, if the degree of ischemia was severer, there might be some morphological differences between the two groups.

The present study has several limitations. Plasma levels of LA was relatively variable between the individuals. It is unclear whether different levels of LA was due to technical inconsistency or individual differences (or even unknown factors). The duration and dose of LA administration have not been investigated. Morphological study was performed using a light microscope as we previously described [1]. However, a more sensitive method might be necessary to detect early changes after ischemia-reperfusion injury, for example, using an electron-microscope. Regarding the ischemic condition, we applied 4 h cold ischemia with crystalloid cardioplegia, which was similar to transplant setting. As future projects, shorter period of ischemia mimicking cardiac surgery and/or warm ischemic setting may be interesting. To assess endothelial function, some scientists use acetylcholine instead of 5-HT. However, we previously reported acute effects of LA on endothelial function using 5-HT, therefore we employed 5-HT also in this study to compare acute and chronic effects of LA [2,3].

In conclusion, the post-ischemic recovery of vascular functions were significantly ameliorated by chronic oral administration of LA in 4 and 8 months rats, but not in 1 and 16 months. Possible cause is that 1 month and 16 months are too immature and too elderly, respectively. Stimulated NO release in response to 5-HT could reflect the better endothelial protection of LA group, therefore, in 4 and 8 months LA fed rats, endothelial and vascular smooth muscle cells were well preserved by stimulated NO release via LA–NO pathway during ischemia-reperfusion. Further studies including mechanistic approach are needed to discover what is immature and how old is the turning point to elderly.

Acknowledgements

We thank Dr Ilona Schmidt and Dr Caroline C. Gray for their technical assistance.

References


