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ORIGINAL ARTICLE

Healthy ageing: the beneficial effect of dietary supplementation with alpha-ketoglutarate on arterial elasticity in elderly mice

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Abstract: The study employed mechanical stretching *in vitro* of sections of abdominal aorta of elderly mice to investigate any benefits of oral treatment with alpha-ketoglutarate (AKG) on arterial elasticity. Eighteen female mice (50-weeks-old) were assigned to a control (2% w/v) Na_2-AKG or (2% w/v) a Ca-AKG group, and treated for 182 days before being humanely killed. Aorta sections were exposed to increases in force (0.09 N) giving approx. 6 kPa of pressure, after which elastic recoil (N ms^{-1} mg^{-1} wet wt.) was measured. Na_2-AKG treatment for 182 days induced a 30% (P <0.01) improvement, while Ca-AKG induced a 93% (P <0.001) improvement in the elastic recoil compared to controls (3.30 $\pm$ 0.08 $\times$ 10^{-5} N ms^{-1} mg^{-1} wet wt). AKG administered orally improved arterial elasticity in elderly mice, a change that occurred despite an increase in total collagen content. Alpha-ketoglutarate warrants further investigation as a candidate for therapies targeting arterial stiffening with age.

Key words: alpha-ketoglutarate, ageing, elasticity, arteries, collagen, blood pressure

INTRODUCTION

It is a well-known fact that men and women are living significantly longer than ever before, with most people pursing a fairly active lifestyle well into their 70's and 80's. However, the process of ageing is associated with a great many changes in body tissues [1], and these can often prove to be potentially fatal.

Atherosclerosis is one of the most important factors involved in the development of hypertension resulting in target organ damage and cardiovascular events [2]. The ensuing hypertension seen with atherosclerosis gives rise to abnormal accumulation of type I and III fibrillar collagens in the walls of arteries, thereby increasing their inherent stiffness [3]. It has recently been shown in a clinical study that arterial stiffening, associated with collagen metabolism in the extracellular matrix, is to be found in hypertensive patients suffering from left ventricular hypertrophy [2]. And yet despite all this, little is known of the functional role played by other collagens, among them collagen VI which has been identified in arterial walls [4].

In another disease often associated with ageing, namely type II diabetes mellitus (DM), a number of studies report an association between atherosclerosis and an alteration in arterial wall stiffness. A rat study in which stiffening of the aortic wall was assessed using intravascular ultrasound, found an increase in aortic wall stiffness in conjunction with an increase in collagen content even at an early stage of pre-diabetes and insulin resistance [5]. More importantly perhaps, is the finding that turnover of collagen accumulated in the aortic wall may become slow with hypertension, and that this slow-turnover may reduce arterial elasticity before morphological changes in the vessel wall can be detected [6]. Whatever the cause of arterial stiffness with ageing, there is a need at present for therapies capable of primarily targeting the stiffness of large arteries, perhaps through inhibition or destruction of cross-linked proteins such as collagen and elastin, as proposed by McEniery and colleagues [7].

Alpha-ketoglutarate (AKG) is known to function as an energy donor and ammonium ion scavenger, as well as providing a source of glutamine that stimulates protein synthesis, inhibits protein degradation in muscle, and constitutes an important metabolic fuel for cells of the gastrointestinal tract. Perhaps of greater importance, however, is the fact that glutamate, synthesized by reductive amination of AKG in peri-vein hepatocytes [8] can give rise to an increase in proline synthesis, which plays a key role in the synthesis of collagen [9, 10]. It has been shown that 7 % of AKG administered enterally but not parenterally is converted to proline, and that paradoxically, the absorption rate for some essential amino acids (e.g lysine, leucine) can be greater than 100% of the level provided in the food when AKG is given as a supplement [11, 12]. The data of Mudge [13], are equally of interest, providing clear evidence of a significant increase in cellular metabolism after exposure to AKG.

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The aforementioned apparently diverse aspects suddenly become of importance when one considers that enterally administered AKG induces proline synthesis [11], that proline plays a key role in the synthesis of collagen [9, 10], and that atherosclerosis/vessel stiffening is associated with the abnormal accumulation of type I and III fibrillar collagens in the walls of arteries [3], where a slow-turnover of collagen appears to reduce arterial elasticity [6]. Thus, it might be postulated that if a slow turnover of collagen really does reduce arterial elasticity, as advocated by Safar and colleagues [6], then a compound that increases cellular metabolism (e.g. AKG) and is associated with the synthesis of collagen, may be beneficial in maintaining a youthful degree of wall elasticity in the elderly.

This study therefore aims to: 1) assess the use and suitability of a simple *in vitro* stretch technique, and 2) address any beneficial effect of AKG-intake with respect to arterial elasticity in the elderly.

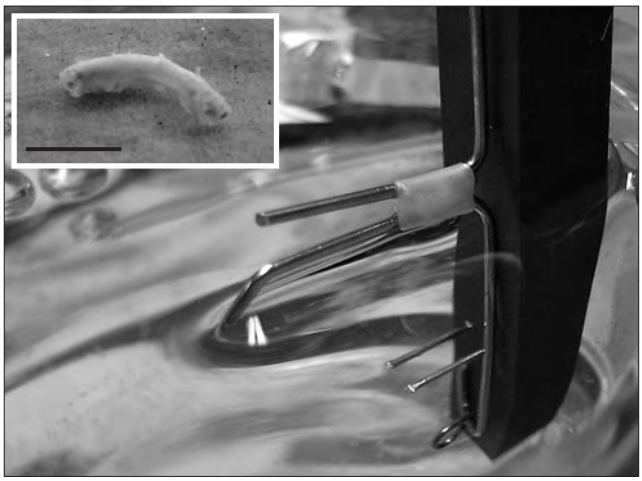

FIGURE 1 A typical mouse aorta section after careful cleaning for extraneous tissue (Insert), scale bar represents 4 mm, and once suspended and fully tensioned, as described in the materials and methods.

MATERIALS AND METHOD

Local ethical permission.

The study was approved by the Ethical Review Committee for Animal Experiments at Lund University (Ethical Allowance M14-05), and was conducted according to European Community regulations concerning the protection of experimental animals.

Animals and aorta preparation. Female NMRI mice, aged 50 weeks at the start of the trial, were housed at the animal facilities of the Department of Cell & Organism Biology at Lund University, Sweden. All animals were raised under the same conditions with a 12/12-hour light-dark cycle. Mice were fed rodent pellets *ad libitum* (Altromin No.1314 Spezialfutterwerke, Lage, Germany), and given free access to drinking water. Mice were randomly allocated to one of 3 groups, and fed for 182 days until they had reached 76 weeks of age, at which time they had attained a body weight of 44.2 ± 1.3 g. The mice in Group 1 were fed rodent pellets plus (2% w/v) Na_2-AKG 2 H_2O (n=6), while the mice in Group 2 were fed rodent pellets plus (2% w/v) Ca-AKG H_2O (n=6). Mice allocated to Group 3, the Control Group, were only fed rodent pellets (n=6). However, the level of AKG fed as a supplement to the diet represented 2% of the voluntary feed intake of the mice, which was approx. 10-15% of body weight per day. No significant difference in body weight was found between the groups.

The mice were anaesthetized by exposure to 95% CO_2, and killed by cervical dislocation. A dissected portion of the abdominal aorta, prior to the right- and left common iliac arteries, was carefully cleaned to remove adhering tissues. The aorta was cut into lengths of approx. 4.5 mm with a diameter at rest of approx.1 mm (approx. area of 14.14 mm^2), and each piece securely attached at one end to a force transducer and at the other to a metal pin on a mounting block, as described in part [14, 15] (Fig 1). On average, the weight of the aorta pieces was 2.75 mg.

Aorta sections were immersed into oxygenated and thermostatically controlled chambers (37°C), having an internal depth and diameter of 5.5 and 3.2 cm, respectively, containing 44 ml of phosphate buffered saline (0.15 M PBS, pH 7.4) comprising in mM; NaCl 136.91, KCl 2.68, Na_2HPO_4 8.08 and NaH_2PO_4 1.66. Force was measured using a FTO3 force displacement transducer (Grass Instrument, West Warwick, RI, USA) connected to a home-built bridge amplifier, which was interfaced with a 8S PowerLab A/D Converter (ADInstruments, Chalgrove, Oxfordshire, UK). The transducer had a functional range of 0-0.05 kg, with a reliable force of 2 mg, equivalent to 0.004% of the functional range. A PowerLab 8S A/D converter was connected to a Book G4 running Chart v.5.4 Software (AD Instruments, Australia). Data recording was performed at a sampling speed of 40.000 data samples per second (40 KHz), and the input impedance of the amplifier was 200 M differential

Force measurements. Aorta sections were suspended vertically and in duplicate. The recorded signal was adjusted to zero for aorta sections without tension with the aid of an offset dial mounted on the pre-amplifier unit. Each aorta section was exposed to a step-wise increase in tension (approx. 0.09 N or 10 g), measured using a FT03 Grass Force transducer. The aorta sections were then allowed to relax totally before being exposed to repeat step-wise increases in tension two more times, in close succession. Aorta sections were subsequently removed and weighed.

Immediately after a step-wise increase in tension, the recording trace was seen to fall very slightly as the aorta tissue exerted a degree of elastic recoil. This fall in the recording trace was measured over time, using the Average Slope calculation available as part of Chart v.5.4 Software (AD Instruments, Australia). Average Slope (g ms^{-1}) is a time derivative of the data points in a trace selection and calculated from the least-square line of best fit.

Elastic recoil calculations. It was assumed that the tension in the wall of the aorta sections was equivalent to that recorded by the force transducer as the result of a manual stretch, The fall in the recording trace seen immediately after a step-wise increase in tension was then measured as a degree of elastic recoil in the aorta sections. The measurement of Average Slope (g ms^{-1}) obtained for each aorta sample was subsequently converted into Newtons (N ms^{-1}) before being adjusted for sample weight to give a final elastic recoil value in N ms^{-1} mg^{-1} wet wt.

Quantitative evaluation of collagen content. Total collagen, and heat soluble collagen, an indirect indicator of cross linkage in collagen (Meyer & Verzár 1959), was determined according to the method of Kristensen et al. (2002). Unless otherwise stated, all chemicals were of analytical grade, obtained from Sigma (Sigma Chemicals Co., St. Louis, MO, USA). Briefly, a sample weighing 0.5-1.5 mg of aorta material (wet wt.) was heated in 100 mM phosphate buffered saline pH 7.2 for 2 h in a circulating water bath at 90°C. After cooling to 40°C, the samples were centrifuged and consequently divided into their supernatant and pellet fractions, respectively. To both fractions, 6 M HCL was added before being hydrolyzed overnight in a sand-bath at 160°C (Harry Gestigkeit GmbH type ST32, Düsseldorf, Germany). Chloramine-T-hydrate was then added to the hydrolyzed preparation, and oxidation allowed to proceed for 25 min. at room temperature. The chromophore was developed with the addition of 1 M Ehrlich's aldehyde reagent, and the Chromophore absorbance was measured at a wavelength of 550 nm. Standard hydroxyprolin samples were processed using the same procedure. The amount of soluble collagen was determined from the hydroxyproline concentration of the supernatant, and the total collagen content was calculated as being the sum of the hydroxyproline concentration of the pellet and that of the supernatant [16].

Statistical analysis. Data are presented as mean ± SE. Differences between means were tested for statistical significance with the use of a one-way ANOVA and an additional test for Gaussian Normal Distribution. Data were found to be normally distributed and to have equal variance. Differences showing a P value >0.05 were considered non-significant.

RESULTS

Tension measurements. An average manual step increase in tension generated 0.09 N or 10 g (4.95×10^{-3} N mg^{-1} wet wt.), and aorta sections were typically found to recoil by 0.015 N or 1.5 g, a value that represents approximately 15-16% of manually applied tension.

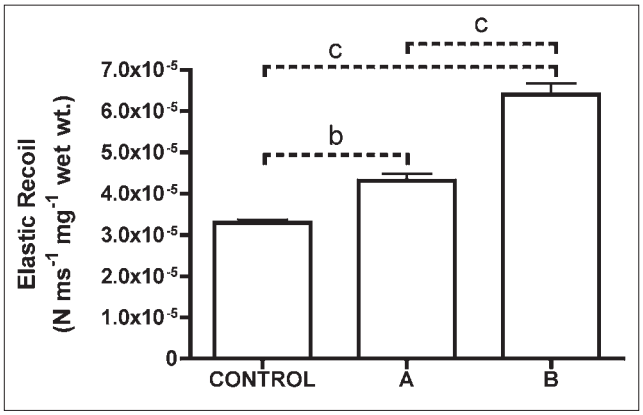

FIGURE 2 First stretch series: Elastic recoil recordings from aorta sections of CONTROL and (A) Na-AKG as well as (B) Ca-AKG treated mice. Recordings were made to a force transducer attached to an A/D converter at a sampling rate of 1,000 samples/s. Each point represents the mean ± SE. Significant differences between the means are given: a = P<0.05 and b = P<0.01, c = P<0.001. Animals allocated to the three groups were n = 6 for all.

Since pressure, measured in Pascals, is defined as Force (N) divided by Area (m^2), the step-wise increase in the force of 0.09N applied over a typical aorta section area of 14.14 mm^2, is equal to a pressure of approximately 6.3 kPa.

Control mice. The elasticity of the aorta for the controls was $3.30 \pm 0.08 \times 10^{-5}$ N ms^{-1} mg^{-1} wet wt. and $3.40 \pm 0.94 \times 10^{-6}$ N ms^{-1} mg^{-1} wet wt. for the first and second series of stretches, respectively. The repeated stretching protocol resulted in approximately a 90% decrease in elastic recoil; second *versus* first series.

Na_2-AKG mice (A). With Na_2-AKG-intake, the elasticity of aorta sections was $4.30 \pm 0.16 \times 10^{-5}$ N ms^{-1} mg^{-1} wet wt. and $3.70 \pm 1.10 \times 10^{-6}$ N ms^{-1} mg^{-1} wet wt. for the first and second series of stretches, respectively. Na_2-AKG-intake also had a significant effect on arterial elasticity compared with the control mice (see Fig 2). The repeated stretching protocol resulted in a 91% decrease in elastic recoil; second *versus* first series.

Ca-AKG mice (B). With Ca-AKG-intake, the elasticity of aorta sections was $6.40 \pm 0.27 \times 10^{-5}$ N ms^{-1} mg^{-1} wet wt. and $3.80 \pm 1.2 \times 10^{-6}$ N ms^{-1} mg^{-1} wet wt. for the first and second series of stretches, respectively. Furthermore, Ca-AKG-intake had a significant effect on arterial elasticity compared with the control mice (see Fig 2). The repeated stretching protocol resulted in a 94% decrease in elastic recoil; second *versus* first series.

Stretch series and arterial robustness. In all the arteries studied, the initial stretch series (e.g. application of tension followed by relaxation) led to a decrease in elasticity with subsequent applications of tension. This effect may be compared to the type of damage that would be expected with a sudden increase in blood pressure (see Fig 3).

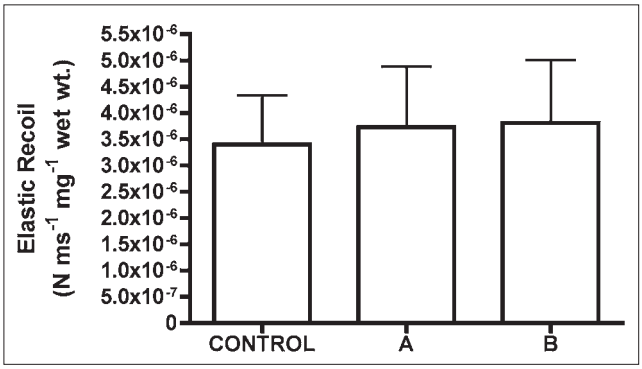

FIGURE 3 Second stretch series: Elastic recoil recordings from aorta sections of CONTROL and (A) Na-AKG as well as (B) Ca-AKG treated mice. Recordings were made to a force transducer attached to an A/D converter at a sampling rate of 1,000 samples/s. Each point represents the mean ± SE Animals allocated to the three groups were n = 6 for all.

Table 1 Robustness of aorta sections to stretch. The number of successful stretches (in triplicate) for excised aorta sections, exposed to a stepwise tension (approx. 0.09 N or 10 g per step) without rupture occurring.

	First Replicate	Second Replicate	Mean (%)
CONTROL	4 out of 6	4 out of 6	66.7
Na_2-AKG (A)	4 out of 6	5 out of 6	75.0
Ca-AKG (B)	4 out of 6	5 out of 6	75.0

Collagen content. An obvious increase in total collagen content was found for both the Na_2-AKG and Ca-AKG treatments, compared with the controls. While neither the Na_2-AKG nor the Ca-AKG differed individually from the control values, there was a significant overall effect of AKG treatment when the data were analyzed using a Kruskal-Wallis nonparametric ANOVA ($P<0.05$; Fig. 4).

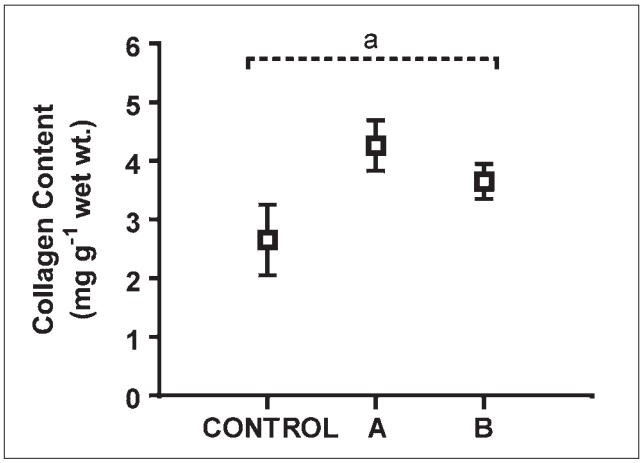

FIGURE 4 Total collagen content. Mouse aorta sections taken from the same samples used for elastic recoil measurements, were additionally analyzed for collagen content (mg g^{-1} wet wt.) using the approach described in the methods section. Each point represents the mean ± SE. Significant differences between the means are given: a = $P<0.05$ – Kruskal-Wallis (Nonparametric ANOVA). Animals allocated to the three groups were n = 6 for all.

A correlation of total collagen content with the elastic recoil data from the aorta sections, however, revealed that whereas there was a clear difference in terms of elastic recoil values between the treatment groups, there was no clear association with total collagen content (Fig. 5), although differences in the relative collagen composition cannot be excluded.

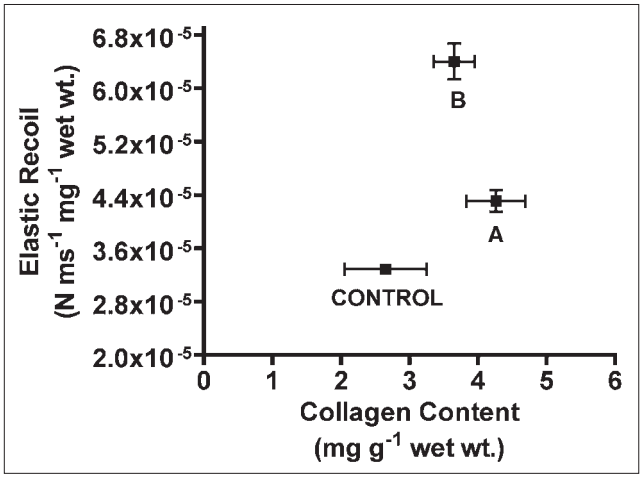

FIGURE 5 Collagen content vs. Elastic recoil. Collagen content values plotted against those for elastic recoil in the first stretch series for identical aorta sections revealed; 1) that as collagen content increased, so too did elastic recoil, and 2) that treatment with (A) Na-AKG was less effective than that of (B) Ca-AKG in terms of elastic recoil, although collagen content was increased equally in both treatment groups. Each point represents the mean ± SE. Animals allocated to the three groups were n = 6 for all.

In support of this, the presented study found that the degree of variation within the groups was less for the Na_2-AKG and Ca-AKG treated animals *cf.* the controls, in terms of the heat-solubility of the total collagen within the aorta sections; 8.96 ± 1.52, 9.42 ± 1.29 and 13.08 ± 2.43 % of total collagen content, respectively. While this 3.7 - 4.1 % reduction in heat solubility for the 2 AKG treated groups was not significantly different from that of the controls, this finding serves to demonstrate that the arterial collagen content was most likely affected in some way.

DISCUSSION

The results of this study clearly show a beneficial effect of alpha-ketoglutarate treatment on arterial elasticity in elderly mice. Moreover, to our knowledge, this is the first time that a therapy capable of targeting the stiffness of large arteries has been reported.

Animals. The animals in this study were chosen as adults with an age comparable to that of an elderly human subject. Upon dissection of the aorta from the mice in this study, it was clear that arterial deposition had taken place so that the aortas appeared almost white to translucent, and even after dissection they retained their tubular shape.

Blood pressure and tension. In rats aged 6 months and older, a blood pressure obtained by cannulating the abdominal aorta or the iliac or carotid artery, with a mean of 119 mm Hg, was recorded (upper limit 150, lower limit 92 mm Hg) [17]. The author of this study also mentions a correlation between age and blood pressure up until 6 months of age, after which no further increase in blood pressure was recorded, despite a further increase in body weight. This value for blood pressure in a small rodent is very close to that of a resting human subject, where the systolic pressure is normally 120 mm Hg (16 kPa). Moreover, the increases in pressure exerted on the artery sections in the present study represent 39 % of that typically found with maximum systolic pressure in human arteries, and approximately 50 % of that measured in rats (13-14 kPa) [18, 19].

Arterial structure/function. The aortic media contains sheets of smooth muscle cells tangentially attached to the elastic lamellae. By varying the distribution of force between the elastic and collagenous fibres, changes in smooth muscle tone provide dynamic, or functional regulation of stiffness [7]. At lower levels of arterial pressure, the resulting stress within the aortic wall is predominantly taken up by the elastin fibres, whereas at higher levels of arterial pressure the stress is generally taken up by the stiffer collagen fibres. Thus, one of the effects of ageing is believed to be the engagement of collagen fibres at lower levels of arterial pressure, concomitantly increasing pulse pressure as a result. One might even go so far as to hypothesize that age-related damage to smooth muscle cells in the wall of the artery results ultimately in the deposition of collagen types I and III in the adventitia, and with them, an increased stiffening of the arterial wall. The development and maintenance of the elasticity of arteries is therefore an important factor in the assessment of a person's risk to succumb to cardiovascular disease.

Ageing arteries. Ageing, which affects organs, tissues and cell types within an organism in different ways, may in many ways be regarded as a differential rate of functional decline [20]. In the vascular wall of large arteries, age-related

structural changes occur, including stiffening and thickening of the media as well as enlargement of the lumen diameter [21, 22], and very often these changes will be heterogeneous along the arterial tree [23-25]. In the aorta of aged rats, modification in smooth muscle cell number, increased collagen deposition, and structural alterations of elastin are characteristic features [22, 26, 27]. Some reports document that the arteries of old rats possess a greater number of smooth muscle cells compared with younger rats [28], while others report a decline in smooth muscle-cell number with age [29, 30], an increase in collagen type I and III, and a relative reduction in elastin density [22, 27, 31]. Collagen VI has been reported as being the major collagen associated with smooth muscle cells (SMC), an aspect of artery elasticity that is potentially of great relevance in terms of the functional properties of ageing arteries [32]. However, to date much of the interest in collagen in the arterial wall has been restricted to collagen types I and III, therefore, we suggest that in the future rather more importance should be given to collagen type VI. Of equal importance is the issue of heat solubility as an indicator of collagen changes, since studies report as high as 25% changes in the heat solubility of total collagen content, associating this with the degree of intermolecular cross-linking of mature collagen fibres [33]. Clearly, the issue of a trend towards a reduction in heat solubility of arterial collagen with AKG treatment in the present study should be investigated further to determine whether it is related to a change in the degree of collagen cross-linkage, and/or changes in collagen type within the various structures of the arterial wall.

It is also worth noting that the stretch approach adopted in the present study revealed a much weaker degree of elastic recoil in the second *cf.* the first series of repeated stretches. This point emphasises the ability, or lack thereof, of elderly aorta sections to cope with a period of relatively mild stretch. Another study has reported a 70% weaker degree of "stiffness" (0.004 N *cf.* the present 0.015 N) in aged rat aortas, but in this particular case a much higher maximum load (1.5-1.8 N) was applied, incurring, one would imagine, a greater degree of damage to the vessel wall [26]. The second stretch may therefore be seen as an index of robustness in the elderly mouse aorta sections. In man, by the age of 60 years, an average individual has experienced more than 2 billion stress cycles of the aorta (average heart rate × age) [7], with damage arising from such stress cycles requiring immediate adjustment and repair involving the elastin, collagen and smooth muscle components of the vessel wall. In the present study, there was no possibility to adjust smooth muscle tone, or any chance of repair of elastin and collagen fibres. Thus, after the first series of stretches, almost 90 % of the elastic recoil (N ms^{-1} mg^{-1} wet wt.) in the control aortas had been lost; similar levels of elastic recoil were also found during the second series for the AKG-treated mice. Overall, this serves to indicate just how vulnerable large vessel recoil is to damage in elderly mice.

Arterial elastic recoil and AKG. While traditional antihypertensive agents have been reported to reduce arterial stiffness, mostly *via* an indirect effect of lowering mean blood pressure, the relative immunity of peripheral arteries to stiffening with age is usually attributed to a much lower ratio of elastin to smooth muscle and to collagen. This, however, may also reflect other biological processes, such as the ability of arteries to remodel themselves [7].

Alpha-ketoglutarate, a rate-determining intermediate in the Krebs cycle, plays a crucial role in cellular energy metabolism. It also functions as a source of glutamate and glutamine, as well as stimulating protein synthesis and inhibiting protein degradation [34]. In terms of collagen metabolism, AKG acts as a co-factor for prolyl-4-hydrolase which catalyzes the formation of 4-hydroxyproline, essential for the formation of the collagen triple helix. Furthermore, AKG also contributes to collagen synthesis through an increase in the pool of proline from glutamate [35] This later action of AKG is supported by our current findings of a significant increase in total collagen content in the 2 treatment groups, compared with that of the controls. In contrast to the rat study, however, in which an increase in aortic wall stiffness in conjunction with an increase in collagen content was reported [36], we found that an AKG-induced increase in arterial collagen content was associated with an improvement in arterial elasticity, and not further stiffening.

AKG has recently been identified as being a natural ligand for a G-protein-coupled-receptor (GPR99), currently known to be expressed in kidney, testis and perhaps most importantly, smooth muscle [37]. As a G-protein-coupled-receptor ligand, AKG might form a link between TCA-cycle intermediates and both metabolic status and protein/collagen synthesis. Indeed, this may well prove to be the underlying cause for the observed beneficial effects on aorta wall elasticity observed in the present study.

It could be postulated that AKG, either acting directly or indirectly, causes activation of arterial smooth muscle cells, resulting in the formation of a highly specific multi-component extra-cellular matrix capable of binding collagen VI and enhancing smooth muscle to smooth muscle interconnections, thereby force production/arterial elasticity. In this way, AKG could be expected to induce an increase in arterial elasticity in elderly subjects. Strangely enough, such an change in extra-cellular matrix and collagen I up-regulation has been reported in smooth muscle cells of the coronary artery upon addition of the growth factor TGF-β1 [38]. The secretion of type VI collagen by macrophages has also been reported to play a functional role in the modulation of cell-cell and cell-matrix interactions [39], findings that lends some support to the possibility of a similar effect of AKG.

Administration of AKG and other tissue effects. Studies using a pig model have shown that AKG given orally is only partly converted to CO_2, compared with glutamine/glutamate, of which 100% is energetically metabolized in its first passage across the intestines (Harrison & Pierzynowski [12]. Lambert et al. [11] subsequently showed that AKG stimulates the gut mucosa into synthesizing proline, the main amino acid required for bone collagen synthesis; thus, oral AKG appears to be much more effective in relation to connective tissue synthesis than glutamine or glutamate. Moreover, proline synthesis can directly affect collagen production *via* its conversion to hydroxyproline, the main amino acid in collagen. This important aspect has been reinforced by the studies of Tatara et al. [40] and Harrison et al. [41], which show that AKG administered orally and not systemically stimulates trabecular collagen synthesis and bone formation.

It might be possible, however, to further enhance the AKG effects reported to date through supplementation of enteral feeding with ornithine α-ketoglutarate (OKG), which has been shown to enhance nitrogen balance and counteract weight-loss [42]. Indeed, parenteral nutrition, including OKG, has been shown to decrease the loss of muscle glutamine that occurs

after surgery [43], and a study involving young rats showed that after only a 2-day period of treatment with OKG a higher plasma glutamine concentration was measurable, compared with that of controls [44]. Finally, a clinical trial with orally administered AKG tablets (calcium salt) given to osteopenia patients, where osteopenia is a subclinical condition with a lower bone fracture rate than osteoporosis, has shown a beneficial reduction in the bone resorption marker CTX, consistent with the preservation of bone mass [45]. Therefore, in terms of healthy ageing, AKG may not only be beneficial in maintaining arterial elasticity, it may also help preserve skeletal mass and alleviate the problems associated with such diseases as osteoporosis.

CONCLUSION

The results of this study, while supporting the conclusion drawn by Koike et al. [46] that "factors responsible for age-associated impairment of angiogenesis are poorly understood", clearly show that an increase in total collagen content with ageing is not always associated with increased arterial stiffening, and that oral administration of the Krebs cycle intermediate AKG may prove an important means of reversing or preventing arterial stiffening in the elderly. On the other hand, working in the last 1950´s, Verzár [47] already had data that supported an exponential age-dependent increase in cross-linkage of collagen fibres. Ageing may thus be associated with a change in collagen type and/collagen structural organization within arterial walls. A further possibility may also be an inherent change in the structure and function of the smooth muscle cells of the vessel walls. Clearly, further investigations are now urgently needed to establish and quantify which collagen types are up- and down-regulated in ageing arteries with AKG treatment, whether AKG specifically regulates certain collagen types during the process of ageing and, to this end, precise and detailed confocal laser scanning microscopy would seem to be an ideal tool in determining exactly where (i.e. smooth muscle *vs.* extra-cellular connective tissue scaffold), different collagen types are associated in the arterial wall. Moreover, in the light of a recent review [12] illustrating the wide-ranging effects of AKG, the effects of such collagen changes in other tissues, organs and systems, e.g. skin, muscle, kidney, bone, etc. now needs to be the focus of future studies.

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AUTHOR CONTRIBUTIONS

All authors contributed towards the compilation and presentation of the manuscript by way of mutually agreeable suggestions and effort.

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