T-kininogen induces endothelial cell proliferation

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Abstract

Basal proliferation of endothelial cells increases with age, and this might play a role in the etiology of age-related vascular diseases, as well as angiogenesis. Serum kininogen levels increase during aging in rats and humans, and T-kininogen (T-KG) can affect proliferative homeostasis in several cell models. Both kinins and kininogens have been shown previously to be angiogenic through activation of endothelial cell proliferation, and here we show that exposure of endothelial cells to T-KG results in vigorous cell proliferation, accompanied by ERK/AKT activation. In our experiments, the proliferative response requires B1 and B2 kinin receptors, even though kinins are not released from the precursor. We hypothesize that the age-related increase in T-KG could play a significant role in the age-related dysregulation of vascular physiology and function.

Keywords: Endothelial cells; T-KG; Kinin receptors; ERK; Cell proliferation; Aging

1. Introduction

Aging is a complex process characterized by a general decline in physiological functions, including significant changes in proliferative homeostasis. While proliferative arrest is the most prominent landmark of a commonly used cellular model of aging, fibroblasts in culture (Cristofalo et al., 1998), not all cell types exhibit a decreased proliferative capacity with age (Holt and Yeh, 1988; Prakash et al., 2002). In some tissues, including the prostate and the vascular endothelium, aging is often accompanied by pathology that includes increased proliferation. Due to its role in haemostatic control and angiogenesis, the vascular endothelium represents a particularly vulnerable tissue (Yu and Chung, 2001; Yeh et al., 2000; Cines et al., 1998).

Impairment of endothelial cell function is believed to contribute significantly to the increased severity of cardiovascular diseases in the geriatric population. In the young adult, endothelial cells are remarkably quiescent (Cines et al., 1998), but they can be easily induced into active proliferation, for example as a result of traumatic injury or inflammation. In contrast, most authors agree that aging results in an increase in the basal level of endothelial cell proliferation (Sholley and Cotran, 1976; Li et al., 1997; McCaffrey et al., 1988). Aorta transplantation experiments between young and old rats suggest that hyper proliferation of vascular smooth muscle cells might be a cell autonomous phenomenon, rather than environment-driven (Hairiri et al., 1986). Similar assessments have not been made with regard to endothelial cells, and while dysregulation has been observed at the level of signal transduction pathways, including Ras/ERK, AKT and Zap 70 (Hoffmann et al., 2001; Hutter et al., 2000; Pahlavani et al., 1998), a role for extrinsic, blood-borne factors has not been ruled out.

Serum T-kininogen (T-KG) levels increase dramatically with age in rats (Sierra et al., 1989, 1992), and a similar observation has been made for HMW-kininogen in both humans (Kleniewski and Czokalo, 1991) and rats (Sierra, 1995), suggesting that this increase is a general phenomenon.
associated with aging. When expressed in BALB/c 3T3 fibroblasts, T-KG inhibits cell proliferation (Torres et al., 2001) and ERK activity (Torres et al., 2000). However, when the same fibroblasts are exposed to exogenous T-KG, both proliferation and ERK activity are increased instead (Aravena et al., 2005). These observations suggest that the age-related increase in serum kininogens might play a role in modulating the changes in proliferative capacity of blood-exposed cells, including endothelial cells. Both HMW-KG and LMW-KG have been shown to be angiogenic, and capable of promoting endothelial cell proliferation and motility (Hayashi et al., 2002; Colman et al., 2003). Furthermore, kininogens are precursors of kinins, vasoactive peptides that also promote endothelial cell proliferation through kinin receptors, primarily B2 (Colman et al., 2003; Seegers et al., 2003). In the case of HMW-KG, release of the kinin moiety leaves behind an entity called HKa, composed of a heavy and a light chain held together by a single disulfide bridge, and which has strong antiangiogenic properties, thus opposing the effect of both kinins and their precursors (reviewed in Guo and Colman, 2005). In contrast, neither LMW-KG nor T-KG gives rise to antiangiogenic moieties after release of their kinins. Thus, these molecules are likely to be only pro-angiogenic in vivo.

We hypothesize that the age-dependent increase in both T-KG and kinins (Pérez et al., 2005) could play a role in the increased endothelial cell proliferation observed in aged individuals. Since endothelial cells do not themselves produce significant amounts of kininogens, we used exogenously added purified T-KG, and our results indicate that T-KG induces endothelial cell proliferation, and this effect is accompanied by an increase in ERK and AKT activities. In contrast, T-KG inhibits the responsiveness of these cells to the stimulus provided by kinins (Leiva-Salcedo et al., 2002). These observations suggest a possible role for serum kininogens in the dysregulation of vascular system physiology and function that occurs during aging, both in rats and in humans.

2. Materials and methods

2.1. Reagents

Antibodies to ERK, P-ERK, AKT and P-AKT were obtained from Cell Signaling. Anti B2 kinin receptor, and horseradish peroxidase-conjugated secondary antibodies were purchased from Transduction Laboratories. Anti-Von Willebrand factor, anti-HMW-KG antibodies, HOE 140, Des-Arg-BK, T-kinin and Bradykinin were from Sigma. The anti-bradykinin antibody used in the RIA was donated by Dr. Kasuaki Shimamoto (Sapporo Medical University, CA) using WinMDI 2.8 software. The number of T-KG receptors and kinetic parameters were obtained through a spectrofluorometric assay, where free T-KG concentrations were measured using a standard curve. Endothelial cells were harvested with PBS/EDTA and 2 × 10^5 cells/ml were incubated with T-KG-FITC (in a range of 0.1–100 μg/ml) for 30 min on ice. The free ligand was separated by centrifugation (10,000 × g, 2 min, 4 °C), and both the pellet and supernatant were adjusted to 110 μl using PBS/SDS 10%. Saturation, non-specific binding and competition assays were performed to establish the specificity of the binding. The kinetics parameters were obtained using Scatchard and Lineweaver–Burk plots (Foreman and Johansen, 2004).

2.2. Purification of T-KG

T-KG was purified from rat serum as previously described (Leiva-Salcedo et al., 2002). Briefly, serum was collected from Brown Norway rats (Katholieken strain) 24 h after LPS injection (2 mg/kg). This strain of rats carries a point mutation in the K-KG gene that eliminates the secretion of both HMW- and LMW-KG into the serum, and thus, T-KG is the only kininogen present in the starting material (Damas, 1996). T-KG was purified from total serum by affinity chromatography on carboxymethyl-papain-agarose. Eluted proteins in the peak at A_{300} were tested for cysteine proteinase inhibitory activity using 10 nM papain and Z-Arg-Arg-NH Mec (Sigma, 1 nM final) as a substrate.

2.3. Expression and purification of recombinant T-KG

The T-KG-GST fusion protein was obtained from Escherichia coli BL21 cells transformed with pGEX-4T constructs encoding T-KG. After induction with IPTG 100 μM for 5 h at an OD_{600} of 0.7, bacteria were pelleted, lysed, sonicated and centrifuged at 14,000 × g, 15 min. The bacterial supernatants were incubated with glutathione–Sepharose (Amersham) for 1 h at 4 °C and GST fusion protein was eluted with 100 mM Tris–HCl pH 8.0 containing 150 mM NaCl and 10 mM glutathione. The eluted proteins were dialyzed into PBS pH 7.4 and T-KG was positively identified using anti-T-KG antibody. cjun-GST fusion protein was used as a negative control.

2.4. Flow cytometry and binding assays

Endothelial cells (1 × 10^5 cells/ml) were incubated on ice with FITC conjugated T-KG (10 μg/ml) for 30 min in the dark at 4 °C in iced-cold PBS containing 0.1% FBS. Cells were then pelleted, washed twice and resuspended in sterile PBS. For competitive FACS analysis, after 10 min of binding to T-KG-FITC, cells were washed and incubated with bradykinin (BK, 100 μg/ml) or unlabeled T-KG (100 μg/ml), for another 30 min. Binding was evaluated by FACscan flow cytometry (Cell Quest, BD Biosciences San Diego, CA) using WinMDI 2.8 software. The number of T-KG receptors and kinetic parameters were obtained through a spectrofluorometric assay, where free T-KG concentrations were measured using a standard curve. Endothelial cells were harvested with PBS/EDTA and 2 × 10^5 cells/ml were incubated with T-KG-FITC (in a range of 0.1–100 μg/ml) for 30 min on ice. The free ligand was separated by centrifugation (10,000 × g, 2 min, 4 °C), and both the pellet and supernatant were adjusted to 110 μl using PBS/SDS 10%. Saturation, non-specific binding and competition assays were performed to establish the specificity of the binding. The kinetics parameters were obtained using Scatchard and Lineweaver–Burk plots (Foreman and Johansen, 2004).

2.5. Cell culture and isolation of primary endothelial cells from rat aorta (RAEC)

Ehly 926 cells (a gift of Dr. Earl Harrison, MCP-Hahnemann University, Philadelphia, PA), were grown in D-MEM containing 10% FBS, 10 U/ml gentamycin (Gibco BRL) and supplemented with HAT in a humidified 5% CO₂ atmosphere at 37 °C.

Rat aorta endothelial cells (RAEC) were purified by a modification of the method described by Suschek et al. (1993). Briefer, aorta from male Sprague-Dawley rats (180 g) was removed, opened longitudinally and the tissue was incubated in D-MEM containing 10% FBS and 100 μg/ml penicillin/streptomycin in the presence of 1 mg/ml collagenase for 30 min at room temperature in an orbital shaker. Then the cells were centrifuged at 1900 × g for 5 min, and the pellet was resuspended in D-MEM containing 10% FBS plus 5% calf serum, heparin (50 μg/ml) and bFGF (10 ng/ml). Cells were seeded on a 60 mm-plate previously coated with fibronectin (10 μg/ml). After 1 h, viable endothelial cells were attached to the matrix, and unattached cells were removed by washing. Cells were further passaged (1:3) every 4 days on dishes previously coated with 2% gelatin, and were used before the 4th passage. RAEC cells were characterized by staining with antibodies against Von Willebrand factor and kinin B2 receptor (Cines et al., 1998). In all experiments, cells were seeded at 1 × 10^5 cells/cm² and grown in a humidified 5% CO₂ atmosphere at 37 °C.

2.6. Preparation of hTERT-RAEC cells

Semi-confluent primary RAEC cells were infected at a m.o.i. of 1:1 with a retroviral vector containing the protein portion of the human telomerase gene (pBNIhEs2-hTERT-Neo, a gift from Dr. C. Sell, Lankenau Institute for Medical
2.7. Cell counting and proliferation assays

Cells were seeded at $1 \times 10^4$ cells/cm$^2$ in 6-well plates, and cultured in D-MEM containing 10% FBS for 24 h. The medium was changed to D-MEM with 0.5% FBS for another 24 h, T-KG (1 $\mu$g/ml) was added and the incubation continued for a further 24 h. The total number of cells was measured at the end of this period in a Coulter counter. For the proliferation assay, cells were seeded in 96-multiwell plates ($1 \times 10^3$ cells/well), and cultured as described above. T-KG (1 $\mu$g/ml, unless otherwise indicated) was added and the incubation continued for a further 18 h, at which time $[^3H]$-TdR (2 nCi/ul) was added and incubation was continued for 6 additional hours. Cells were lysed with 10% trichloroacetic acid (TCA). The acid-insoluble material was collected on glass microfiber filters (GF-C), washed with 5% TCA and ethanol, and then the filters were dried and counted in triplicate in a liquid scintillation counter (Tracor Analytic Delta 300).

2.8. Kinin radio immuno assay (RIA)

Free kinins present in the cell supernatants were measured as described (Pérez et al., 2005).

2.9. Statistical analysis

All experiments were done at least in triplicate. Data are expressed as mean ± S.E. and were analyzed by the non-parametric test of Mann–Whitney. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Exogenous T-KG induces endothelial cell proliferation

To test the possible effects of exogenous T-kininogen on endothelial cell proliferation, EAhy 926 cells were serum starved for 24 h and then, a variable amount of purified T-KG was added to the medium for 24 h. The concentration of T-KG used (0.25–6.6 $\mu$g/ml) is approximately 2 orders of magnitude below the physiological concentration of the protein in normal rat serum (Raymond et al., 1996). However, most of this T-KG exists in the serum as a multimolecular complex (E.L. and F.S., unpublished observations), and we estimate that the concentration of free T-KG in old animals is only approximately 10–20 $\mu$g/ml, close to the range of concentrations used here. Incorporation of $[^3H]$-TdR was assessed during the last 6 h of incubation by measuring TCA precipitable counts. Fig. 1A indicates that exogenous T-KG induces $[^3H]$-TdR incorporation in these cells in a strictly dose-dependent manner, up to a concentration of 1 $\mu$g/ml. This concentration of T-KG was used in all subsequent experiments. To test that this really represents cell proliferation, we did direct cell counting, 24 h after addition of 1 $\mu$g/ml of purified T-KG. Fig. 1B shows that T-KG increased the number of cells to almost twice, relative to control. To confirm that this was not due to a contaminant in the preparation, Fig. 1B shows that a similar effect is obtained in the presence of recombinant T-KG-GST fusion protein (1 $\mu$g/ml), but not when we used cjun-GST fusion protein as a control. Fig. 1C indicates that the effect is not unique to EAhy 926 hybridoma cells, since an approximately 2–3-fold increase in $[^3H]$-TdR incorporation was also observed in primary cultures of rat aorta cells (RAEC), and in hTERT-RAEC, a stable cell line prepared by retroviral infection of rat aorta endothelial cells with hTERT. The hTERT-RAEC cells retain a differentiated phenotype, as assessed by staining with anti-Von Willebrand factor, as well as anti-B2 kinin receptors (data not shown). Exposure of hTERT-RAEC cells to 1 $\mu$g/ml T-KG leads to an increase in $[^3H]$-TdR incorporation comparable to that observed in the other cell types, and also quantitatively
comparable to that observed after serum stimulation of quiescent hTERT-RAEC cells (Fig. 1C). Therefore, all subsequent experiments were performed in hTERT-RAEC cells, since they represent a more easily manipulated functional model of endothelial cells.

3.2. T-KG binds to endothelial cells

As a first approach to explore the effect of T-KG on endothelial cells, we measured its binding at 4 °C, using flow cytometry. Fig. 2A shows that T-KG (10 μg/ml) binds to endothelial cells, and this binding is completely displaced by unconjugated T-KG (100 μg/ml) or by BK (100 μg/ml). Furthermore, Fig. 2B shows that binding of T-KG to endothelial cells is dose-dependent and saturable (Fig. 2B inset). A Scatchard plot (Fig. 2B) suggests the presence of two classes of binding sites. Using the Lineweaver–Burk plot of reciprocal doubles, we find that hTERT-RAEC endothelial cells have approximately 74.6 ± 19.6 high affinity binding sites/cell, with a dissociation constant of 10.8 ± 3.8 μM. Low affinity binding sites (approximately 600 ± 44 binding sites/cell, with a \( K_d = 34.3 ± 8 \) μM), were also detected.

3.3. The induction of endothelial cell proliferation depends on the time of exposure to exogenous T-KG

To determine the minimal time necessary for T-KG to exert its action, we incubated hTERT-RAEC cells for different times in the presence of 1 μg/ml T-KG. After washing the cells twice to eliminate any unbound T-KG, cultures were replenished with 0.5% FBS-containing medium, and incubation was continued for a total of 24 h from the initial addition of T-KG. \( ^{3} \text{H} \)-TdR incorporation was measured during the last 6 h of incubation as described above. (A) Schematic representation of the experimental approach. (B) Cell proliferation was measured as described. The results are expressed relative to the control (Co), which received no T-KG, but was treated exactly as the experimental samples (\(* p < 0.05\)).

3.4. Induction of cell proliferation requires both types of kinin receptors

T-KG is a precursor of T-kinin, and it has been shown to bind as such to kinin receptors (Morbidelli et al., 1998). However, its effect on these receptors is not known. To assess whether or not the effects of T-KG on cell proliferation are mediated by kinin receptors, we measured cell proliferation in the presence of 2 μM HOE 140 (a B2 receptor antagonist) or 1 μM Des-Arg\(^{9}\)-leu\(^{8}\)-BK (a B1 receptor antagonist). The
results shown in Fig. 4A indicate that cell proliferation in response to exogenous addition of T-KG requires both types of receptor. As a control, when used alone, the antagonists by themselves did not have a significant effect on proliferation.

Since kinins are known to induce endothelial cell proliferation (Seegers et al., 2003), one possible explanation for our results is that T-kinin might have been released into the culture medium from the precursor T-KG. By using a sensitive RIA assay to measure free kinins in the culture medium, we were unable to detect an increase in kinin levels in the T-KG-containing conditioned medium (Fig. 4B). Because of the extreme instability of kinins, these measurements were done at different times, and kinins were stabilized using ramiprilat, an ACE inhibitor that also strongly inhibits kininase activities (kinin-degrading enzymes). Stabilization after collection was also attempted by using an ethanol precipitation protocol (Pueyo et al., 1996), but still, we detected no T-KG-dependent increase in kinins in the ethanol supernatants (Fig. 4B). Furthermore, Fig. 4C indicates that neither the MW, nor the mass of the added T-KG is affected by its addition to the culture medium. Release of T-kinin would have decreased the MW of the precursor by approximately 10 kDa. Therefore, we conclude that T-kinin is not released from its precursor under our experimental conditions, and thus the proliferative effects we observe are due to T-KG itself.

3.5. The induction of cell proliferation requires several signal transduction pathways

Binding of T-KG to the cells is competed by free kinins (Fig. 2) and the proliferative effect requires kinin receptors (Fig. 4A). Kinin receptors have been shown to activate a large network of signal transduction pathways, and we used a pharmacological approach to establish which pathways are required for the proliferative effect. In all cases, inhibitors were added 30 min before T-KG, and proliferation was then measured 24 h later as described. As a control, we assessed toxicity of the inhibitors by cell counting at the end of the experiment. None of the treatments induced cell loss by more than 10%, compared to FBS alone (data not shown). The results shown in Fig. 5 indicate that the induction of cell proliferation by exogenous T-KG requires, at the least, ERK, PI3K, PKC and PKA. These results suggest a complex mechanism of induction of cell proliferation, that includes the kinin receptors, and activation of several signal transduction pathways known to be located downstream of the receptor: ligand binding event.
3.6. Exogenous T-KG induces AKT and ERK activation in endothelial cells

The ERK and AKT pathways are the signal transduction mechanisms most traditionally associated with induction of cell migration and proliferation, and inhibition of either of these pathways eliminates the proliferative effect of T-KG. Therefore, it was of interest to establish the kinetics of activation of these two pathways in response to T-KG exposure. We observed activation of AKT, showing a single peak at 15 min after T-KG addition (Fig. 6A). We also observed a rapid biphasic activation of ERK, with a first peak at 5 min after T-KG addition, and a second, lower but sustained peak that starts at approximately 60–120 min (Fig. 6B). Because this second peak is not observed when endothelial cells are induced with kinins (Leiva-Salcedo et al., 2002), we chose to assess its relative importance for the proliferative effect. For this, we repeated the proliferative assay in the presence of PD098056. However, this time the inhibitor was added 15 min after the addition of T-KG (i.e., after the first peak of ERK activity). The results shown in Fig. 7 indicate that under these conditions, the proliferative effect of T-KG is completely abrogated, suggesting that the second peak of ERK activity is important and necessary for the proliferative effect of T-KG on endothelial cells. This observation provides further evidence suggesting that the proliferative effect of T-KG is not mediated by the release of free kinins. Furthermore, this result is consistent with data presented in Fig. 3, showing that a short exposure of cells to T-KG is not sufficient to trigger cell proliferation.

4. Discussion

Endothelial cell function declines with age, leading to a functional decline in several hemodynamic parameters (Marin, 1995; Challah et al., 1997). Among the age-related changes in vascular physiology, increased endothelial cell proliferation can lead to hyperplasia as well as angiogenesis. Several reports have indicated an increased turnover of both smooth muscle and endothelial cells in regions susceptible to atherosclerosis (Okuda et al., 2000; Minamino et al., 2002). Phillips et al. (1994) described that primary endothelial cells derived from old rats respond better than those from young rats to the proliferative stimulus of serum, while Challah et al. (1997) described an increase in the number of circulating endothelial cells in old rats, suggesting an increase in the rate of endothelial cell turnover with aging. These findings were not confirmed by
Cortés et al. (2002) who found that arterial cell proliferation was similar in young and old animals. The mechanisms responsible for this age-related increase in proliferation are not known, and our results suggest that the increase in serum levels of kininogens observed in old rats and humans could play a role. Interestingly, we have previously shown that expression of T-KG in mouse fibroblasts inhibits cell proliferation (Torres et al., 2000, 2001), while exogenous addition of T-KG leads to increased proliferation instead (Aravena et al., 2005). Thus, at least in fibroblasts, the effect of T-KG can be diametrically opposite, depending exclusively on the mode of administration (endogenous expression versus exogenous addition).

It has been described that both kininogens and their metabolite, kinins, can induce angiogenesis and endothelial cell proliferation (Morbidelli et al., 1998; Parenti et al., 2001). The data presented in Fig. 4 suggests that free kinins are not produced to a large extent in our system. This is not surprising, since T-KG is a unique kininogen, which is not susceptible to tissue kallikreins present in endothelial cells, and T-kininogenases have not been described in these cells. Furthermore, the proliferative effect of kinins has only been observed at high concentrations, and even if all the T-KG present in our experiments was fully converted to T-kinin, the level of the peptide would only reach 15 nM, a concentration at least one order of magnitude lower than that used to induce proliferation. Finally, the activation of ERK by T-KG (Fig. 6) is kinetically different from that elicited by kinins, and the proliferative effect induced by T-KG requires the second peak of ERK activation, which is not present in kinin-treated cells. In fact, we have shown that T-KG actually inhibits ERK activation in response to kinins (Leiva-Salcedo et al., 2002; Pérez et al., 2005), probably through competition for the same receptors in the cell surface. Taken together, it appears most likely that T-KG, like its counterparts HMW-KG and LMW-KG, induces endothelial cell proliferation as such, without needing to release kinins. The fact that this effect requires kinin receptors is consistent with reports indicating that HMW-KG (the precursor of bradykinin) can bind to these receptors, at least in vitro (Hasan et al., 1994).

Binding of kinins to either of its receptors leads to the activation of a complex array of signal transduction processes. Consistent with this, our studies indicate that the proliferative effect of T-KG requires, at the least, the activity of ERK, AKT, PKC and PKA. It should be mentioned that the parameter we have measured, proliferation after 24 h, is complex in nature and could well require the coordinate activity of all these pathways. Using the SW-480 cell line, Graness et al. (1998) observed that the proliferative response induced by bradykinin involves PKCα, PI3K and ERK, and the authors suggest the involvement of a trans-activation mechanism for the activation of ERK (Marinissen and Gutkind, 2001). Indeed, many G protein coupled receptors appear to activate the ERK pathway indirectly, through a mechanism that requires a membrane metalloprotease, which activates tyrosine kinase-containing receptors (Zwick et al., 1999; Mukhin et al., 2003). Intriguingly, we have previously shown (Aravena et al., 2005) that in fibroblasts, the induction of cell proliferation by exogenous T-KG is independent of kinin receptors, as determined by susceptibility to the same antagonists used here. It is therefore likely that these differences might be due to variation in the number and type of receptors each cell type expresses on its surface.

The data presented here suggests that the age-related increase in serum T-KG levels could lead to an increase in the proliferative capacity of endothelial cells, thus potentially playing a role in angiogenesis in the old. In considering the physiological implications of our findings, it is worth mentioning that the amount of exogenous T-KG we have used in our experiments is well below physiological concentrations (normally 1255 ± 57 μg/ml of blood in young rats (Raymond et al., 1996). Thus, our experiments are not done under pharmacological conditions. On the other hand, the effects we have observed were obtained with concentrations that are so much below physiological, that it is possible to argue against their relevance from that side as well. We would argue that the in vivo concentration of free T-KG (that is, T-KG not associated in macromolecular complexes) is close to the concentration used here, and it is only this free T-KG that has the biological activity. Indeed, formation of multimolecular complexes in vivo could buffer the physiological effects of T-KG. Our unpublished data (E.L. and F.S., unpublished observations) indicate that free T-KG does indeed exist in the serum of old Fisher 344 rats, to a larger extent than observed in younger animals. In all cases, however, free T-KG appears to represent only a small percentage of the total (between 1 and 5% in different animals), and therefore, the concentration of free T-KG in rat serum is likely to be within the range used in these experiments. Thus, at least in old individuals, it is likely that free T-KG can play a role in the observed increase in proliferation of endothelial cells. We propose that, through its effects on endothelial cells, serum T-KG might influence vascular functions, including atherosclerotic plaque formation and increased angiogenesis. Since we have previously shown that serum T-KG levels increase with age in several strains of rats, our results further imply this molecule as a relevant player in age-related vascular pathology.

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