T-kininogen can either induce or inhibit proliferation in Balb/c 3T3 fibroblasts, depending on the route of administration

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Abstract

T-kininogen (T-KG) is a precursor of T-kinin, the most abundant kinin in rat serum, and also acts as a strong and specific cysteine proteinase inhibitor. Its expression is strongly induced during aging in rats, and expression of T-KG in Balb/c 3T3 fibroblasts results in inhibition of cell proliferation. However, T-KG is a serum protein produced primarily in the liver, and thus, most cells are only exposed to the protein from the outside. To test the effect of T-KG on fibroblasts exposed to exogenous T-KG, we purified the protein from the serum of K-kininogen-deficient Katholiek rats. In contrast to the results obtained by transfection, exposure of Balb/c 3T3 fibroblasts to exogenously added T-KG leads to a dose-dependent increase in [^{3}H]-thymidine incorporation. This response does not require kinin receptors, but it is clearly mediated by activation of the ERK pathway. As a control, we repeated the transfection experiments, using a different promoter. The results are consistent with our published data showing that, under these circumstances, T-KG inhibits cell proliferation. We conclude that T-KG exerts opposite effects on fibroblast proliferation, depending exclusively on the way that it is administered to the cells (transfection versus exogenous addition).

Keywords: Aging; Kininogen; Fibroblasts; Proliferation; Signal transduction

1. Introduction

T-kininogen (T-KG) is a multifunctional protein characterized primarily as a precursor of the vasoactive peptide T-kinin and as a potent physiological inhibitor of cysteine proteinases (Anderson and Heath, 1985). Hepatic expression of the T-KG gene increases dramatically towards the end of lifespan in rats of different strains and of both sexes (Sierra et al., 1989; Walter et al., 1998). This in turn leads to an increase in serum levels of the protein (Sierra et al., 1992), and we have recently shown that serum kinin levels are also increased in parallel (V. Pérez et al., in preparation). Through differential splicing, the other major kininogen in the rat, K-kininogen, gives rise to both HMW- and LMW-KG (Kakizuka et al., 1988), and we have also previously shown an age-related increase in serum levels of HMW-KG in rats (Sierra, 1995). Similar observations have been made for the human ortholog, HMW-KG (Kleniewski and Czokalo, 1991). In order to examine the physiological role of the increase in T-KG during aging, we have previously expressed the protein in several fibroblast cell lines. When using the strong constitutive CMV promoter, T-KG expression was incompatible with cell growth (unpublished data), while more modest expression under control of the metallothionein-1 promoter resulted in a strong inhibition of proliferation in both Balb/c 3T3 and L_{TK-} fibroblasts (Torres et al., 2001). This inhibition was accompanied by a decrease in basal ERK activity, apparently as a result of T-KG-

Abbreviations: ERK, extracellular signal-regulated kinase; [^{3}H]-TdR, [3H]-thymidine; HMW-KG, high-molecular-weight kininogen; LMW-KG, low-molecular-weight kininogen; T-KG, T-kininogen

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dependent stabilization of the phosphatase MKP-1, a key enzyme involved in the negative control of ERK activity (Torres et al., 2000, 2001).

The liver is the main site of T-KG synthesis, and most other cells do not normally express the protein (Chao et al., 1988). However, like many other liver proteins, T-KG is efficiently secreted into the bloodstream, where it can interact with a variety of cell types, including lymphocytes, macrophages and endothelial cells. Under pathological conditions, both breakage of the endothelial barrier or transvasation can lead to blood components to interact with fibroblasts as well. In fact, fibroblast migration and proliferation in the aorta have been described as important events in cases of restenosis, as well as atheromatosis (Shi et al., 1996; Roy-Chaudhury et al., 2001). Therefore, investigation of the effect of exogenous administration of T-KG to these cells becomes relevant if we are to understand the role of the increase in serum T-KG levels in age-related pathology and physiology. In order to investigate whether exogenous T-KG can exert a similar inhibitory function when applied from the outside of the cell, we have undertaken the purification of native T-KG from LPS-inflamed Brown Norway Katholiek rats (Leiva-Salcedo et al., 2002). This rat strain is characterized by a single point mutation that impedes the secretion of K-kininogen into the bloodstream (Damas, 1996). Thus, the only known kininogen present in the serum of these rats is T-KG. Exogenous administration of purified biologically active T-KG to normal Balb/c 3T3 fibroblasts results in a reproducible dose-dependent induction of [^{3}H]-TdR incorporation, a result diametrically opposed to our previous observations using endogenous expression of the protein. The results are not due to changes in the cell lines under study, since entry into S phase was still inhibited if T-KG was expressed endogenously in the same cells. Since the concentration of T-KG present in the conditioned medium is similar under both conditions, we conclude that the effect of T-KG on fibroblast proliferation is strongly dependent on the way the protein is administered to the cell.

2. Materials and methods

2.1. Purification of T-KG

T-KG was prepared as previously described from the serum of Brown Norway Katholiek rats (Leiva-Salcedo et al., 2002). This strain does not secrete either the high- or low-molecular-weight isoforms of kininogens (HMW-KG and LMW-KG, respectively), thus yielding a cleaner preparation of T-KG. The purity of the preparations has been assessed by both silver staining and Western blot. Major likely contaminants (such as serum albumin) have been ruled out by enzymatic digestion of the purified material with several proteases, and biological activity was assessed by measuring cysteine protease inhibitory activity by means of a direct assay, using 10 nM papain as a substrate (Leiva-Salcedo et al., 2002). Inhibition was observed at equimolar rates, suggesting full activity recovery.

2.2. Cell culture

Balb/c 3T3 fibroblasts were routinely maintained in D-MEM supplemented with 10% FBS in the presence of 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cell lines expressing T-KG were prepared using the LAP (Lac I activator protein)-IPTG reversible gene expression system (Miao and Curran, 1994; Labow et al., 1990). Balb/c 3T3 fibroblasts expressing the LAP protein (Blap-1, encoding the IPTG-repressible LAP protein under the control of the human β-actin promoter) were co-transfected with plasmids pLap-Kin (containing a full-length T-KG cDNA under the control of three copies of the *lac* operator sequence), and pBabePuro (Morgenstern and Land, 1990) for selection. Stable clones were selected in 5 μg/ml puromycin. Cells were grown in the absence of IPTG and expression of T-KG was determined by Western blot analysis of total cell extracts.

Cells were routinely seeded at 3×10^3 cells/cm^2 and grown for 24 h under standard conditions before experimentation. In the case of synchronized cultures, cells were washed twice in warm PBS, and cultured for an additional 2 days in D-MEM with 0.5% FBS before addition of exogenous T-KG. Exogenous T-KG was diluted in D-MEM and added to the cells at the concentration indicated in each figure, but usually at 2 μg/ml.

2.3. DNA replication assay

Cells were seeded in 96-well polystyrene plates (Nunc International, Denmark) and cultured as described. DNA replication was assessed 24 h after seeding by adding [^{3}H]-TdR to a final concentration of 4 μCi/ml and the incubation was continued for an additional 4 or 12 h. Then the cells were lysed, fixed with 10% TCA and the acid-insoluble material was collected on glass microfiber filters (Whatman International Ltd., England). Radioactivity was quantified using a liquid scintillation counter. When necessary, purified T-KG was added at different concentrations, 12 h before [^{3}H]-TdR addition, for a total incubation time of 24 h.

2.4. Western blot analysis

Total cell extracts were obtained by washing cell monolayers twice in ice-cold PBS, followed by lysis in Laemmli buffer containing 100 mM β-mercaptoethanol at 90 °C. Extracts were sonicated twice at 75 W, 22.5 kHz, 15 s in an ice-cold bath and soluble material was stored at −20 °C until use. Proteins were separated by 10% SDS-PAGE, and electrotransferred to nitrocellulose membranes (Schleicher & Schuell). Western blotting was performed

under standard conditions using horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse IgG as secondary antibodies, and the ECL system (Amersham Biosciences) for detection. In all cases, data were normalized to β-actin abundance by stripping and re-probing the membranes with anti β-actin monoclonal antibody (ICN) as a control for gel loading.

2.5. Pharmacological studies

Antagonists of the kinin receptors and specific inhibitors were prepared in D-MEM without serum at the following final concentrations: 1 μM HOE-140, 1 μM Des-Arg^9-leu^8-BK, 50 μM PD098056, 1 μM H89 and 100 nM bis-indoleylmaleimide. Culture medium was replaced by these solutions 30 min before induction with 2 μg/ml T-KG, and incubation was continued for 24 h. [^{3}H]-TdR was added 12 h after T-KG addition. T-KG plus DMSO and DMSO alone were used as controls.

2.6. Statistical analysis

For all experiments, measurements were done at least in triplicate, and data are expressed as means ± S.E.M. Statistical significance was established using a one-tailed non-parametric Student's *t*-test.

3. Results

3.1. Expression of T-KG in Balb/c 3T3 fibroblasts inhibits [^{3}H]-TdR incorporation

In order to confirm our previous observations showing that a low basal level of T-KG expression can inhibit proliferation of Balb/c 3T3 fibroblasts, we transfected BLap-1 cells with a construct containing the T-KG cDNA under the control of the *lac*I operator sequence, which responds to binding of the *lac*I activator protein, LAP (Labow et al., 1990). BLap-1 are modified Balb/c 3T3 cells that only produce LAP and served as the parental line. In transient assays, immunofluorescence indicated production of very high amounts of T-KG. However, most of these cells failed to proliferate, few clones were obtained after selection, and of the colonies observed, most collapsed before analysis was possible (data not shown). These results are consistent with our previous studies expressing T-KG under the control of the CMV promoter (unpublished observations). We then prepared several new cell lines using the same constructs. As indicated in Fig. 1A, two different clones that express T-KG of the expected molecular mass (68 kDa, clones LapKinC_{10} and C_4) produced the highest levels of immunoreactive protein. $B_{RTK\text{-}5}$ and Balb/c 3T3 cell extracts were used as positive and negative controls, respectively. $B_{RTK\text{-}5}$ cells correspond to a Balb/c cell line co-transfected with Ha-Ras and T-KG under the control of the CMV promoter. Thus,

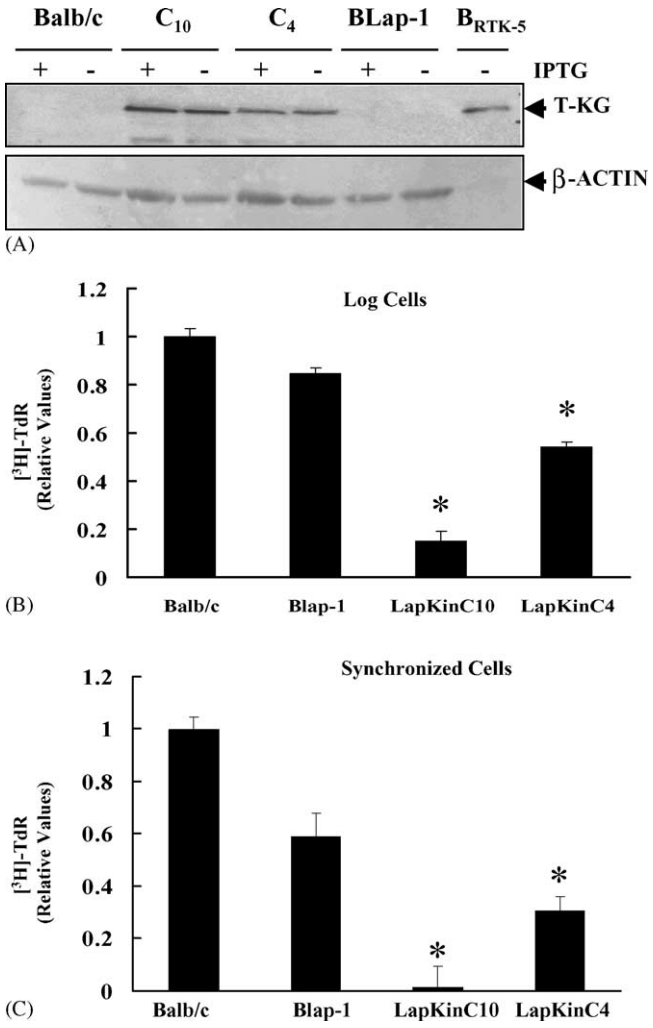

Fig. 1. T-KG expression in Balb/c 3T3 fibroblasts inhibits entry into S phase. (A) T-KG expression by different cell lines was assessed by Western blot analysis of total cell extracts prepared 24 h after seeding, either in the presence (+) or absence (−) of 10 nM IPTG. Balb/c 3T3 (labeled Balb/c) are the parental cells, BLap-1 are cells transfected with vector alone, and $B_{RTK\text{-}5}$ is a positive control, transfected with both T-KG and Ha-Ras (loaded at 1/10 of the protein present in other lanes). C_4 (LapKin C_4) and C10 (LapKin C_{10}) are two cell lines prepared as described in Section 2. β-Actin levels were measured as a loading control. (B) Cells were seeded, and 24 h later, an aliquot of [^{3}H]-TdR was added. Twelve hours later, the amount of radioactivity incorporation was measured as described in Section 2. (C) After 24 h in culture, cells were serum deprived for 48 h, and then serum (10%) was added for an additional 24 h. Incorporation of [^{3}H]-TdR was measured during the last 12 h of this period. Incorporation is given relative to that observed in Balb/c 3T3 cells. All measurements were done in triplicate, and the experiments were repeated at least three times. In (B) and (C), graphs represent the mean ± S.E.M. Asterisks denote statistically significant differences ($P < 0.01$) relative to BLap-1 cells.

these cells express very high levels of T-KG (unpublished data). Unexpectedly, we observed that T-KG expression was not particularly inducible by removal of IPTG (which in this case acts as a repressor of T-KG expression). However, the isolated cell lines have two critical features of interest for our further studies: (1) they express T-KG constitutively and (2)

they grow very slowly, as estimated by their reduced passage frequency (data not shown).

The data from [^{3}H]-TdR incorporation assays shown in Figs. 1B and C indicate that expression of T-KG leads to a significant inhibition of entry into S phase, both in logarithmically growing cells (Fig. 1B) and in cells synchronized in G_0 by serum deprivation and then induced to enter the cell cycle by addition of serum (Fig. 1C). The degree of inhibition was proportional to the amount of T-KG expressed in each of the two cell lines analyzed. These results are consistent with our previous studies in fibroblast cell lines that express T-KG under the control of the MT-1 promoter (Torres et al., 2001).

3.2. Exogenous T-KG stimulates entry into S phase

Our data, both published and as shown in Fig. 1, indicate that overexpression of T-KG in fibroblasts leads to inhibition of cell proliferation. Because fibroblasts do not normally produce this protein, but can be exposed to it under certain pathological conditions, we wanted to test if exogenous T-KG can exert similar effects. In overexpression experiments, inhibition apparently occurs at or near the G_1/S interphase (Torres et al., 2001). Thus, we investigated the effect of exogenous T-KG on entry of normal untransfected fibroblasts into S phase. With this purpose, we synchronized the cells in G_0 by serum deprivation and cells were then preincubated for 12 h in the presence of increasing concentrations of purified T-KG. At the end of this period, we induced a proliferative response by addition of 10% FBS in the presence or absence of T-KG, and the incubation was continued for different times. DNA synthesis was assessed by adding radioactive nucleotide during the last 4 h period, as described in Section 2. Fig. 2 shows that T-KG treatment induced a slight increment in [^{3}H]-TdR incorporation, but without modifying the kinetics of entry into S phase. This effect was modest, but was also contrary to our expectations. More surprisingly, however, the data also indicate that exogenous T-KG stimulates entry into S phase at about 4 h post-serum-addition. This effect is too fast to be related to the serum response, but it occurs approximately 16 h after the initial addition of T-KG, suggesting that it might be due to an effect of T-KG, rather than serum. This peak in DNA synthesis is strictly dose-dependent.

3.3. Exogenous T-KG induces [^{3}H]-TdR incorporation in normal untransfected Balb/c 3T3 fibroblasts

The results shown in Fig. 2 suggest that T-KG exerts an S-phase stimulatory effect when it is externally administered to Balb/c 3T3 fibroblasts. Since we observed this effect shortly after serum addition, we repeated the experiment in the absence of this proliferative stimulus. Fig. 3 indicates that exogenous T-KG can induce [^{3}H]-TdR incorporation in fibroblasts, both in logarithmically growing cells (Fig. 3A) and in cells synchronized in G_0 by serum deprivation

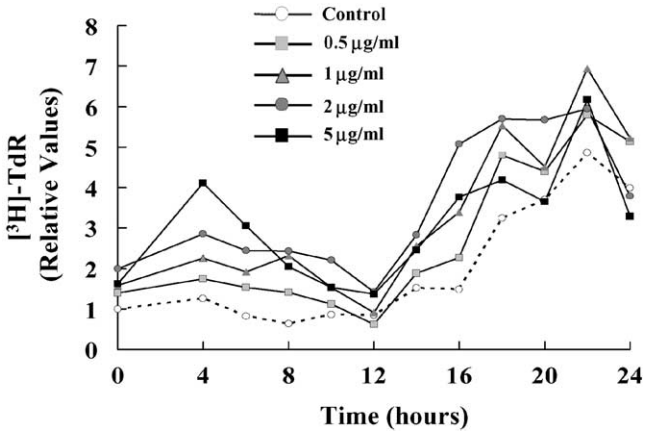

Fig. 2. Entry into S phase in response to serum in the presence of exogenous T-KG. Balb/c 3T3 fibroblasts were seeded and synchronized in G_0 as described in Fig. 1. After 2 days of serum deprivation, T-KG was added to a final concentration between 0 and 5 µg/ml. Serum (10%) was added 12 h later. [^{3}H]-TdR was added at different times after serum addition and incorporation was measured at 4 h intervals during the following 24 h, as described in Section 2. Incorporation is given relative to that observed at time zero in control cells. Times indicated refer to the time of sample collection. The experiment was performed only once, with all measurements done in triplicate.

(Fig. 3B). The effect is not particularly robust, reaching approximately a two-fold increase in [^{3}H]-TdR incorporation when T-KG is given at 2 µg/ml. This stimulation is, however, dose-dependent and highly reproducible ($P < 0.01$).

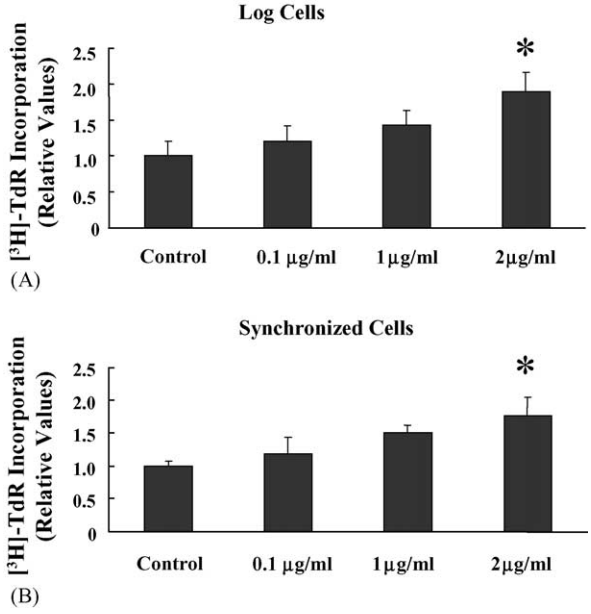

Fig. 3. T-KG induces fibroblast entry into S phase. Cells were seeded and either synchronized (panel B) or not synchronized (panel A) by serum deprivation, as described in Fig. 1. T-KG was added 48 h after either seeding (panel A) or serum deprivation (panel B), and [^{3}H]-TdR was added 12 h after T-KG addition. Incubation was continued for a further 12 h. All measurements were done in triplicate, and the experiments repeated at least three times. Graphs represent the mean ± S.E.M., and data are presented relative to the controls without addition of T-KG. Asterisks denote significant differences ($P < 0.01$) relative to these controls.

3.4. T-KG activates signal transduction pathways, but not through the kinin receptors

T-KG is a precursor of T-kinin, which can exert a proliferative effect in different cell types through activation of B1 or B2 kinin receptors, followed by activation of several signal transduction pathways (reviewed in Campbell, 1995). In an effort to determine the molecular mechanisms involved in the activation of fibroblasts by exogenous T-KG, we used kinin receptor antagonists and several pharmacological inhibitors of some important signal transduction pathways involved in the proliferative response of fibroblasts. The results shown in Fig. 4A indicate that entry into S phase in response to exogenous addition of T-KG is not abrogated by either of the specific kinin receptor antagonists used, HOE140 (B2 receptor antagonist) or Des-Arg^9-leu^8-BK (B1 receptor antagonist). Thus, the stimulatory effect of exogenous T-KG does not require kinin receptors, and indeed, does not seem to require the release of T-kinin (see below). In fact, the stimulatory effect appears to be due to T-KG itself, probably by its interacting with another surface receptor.

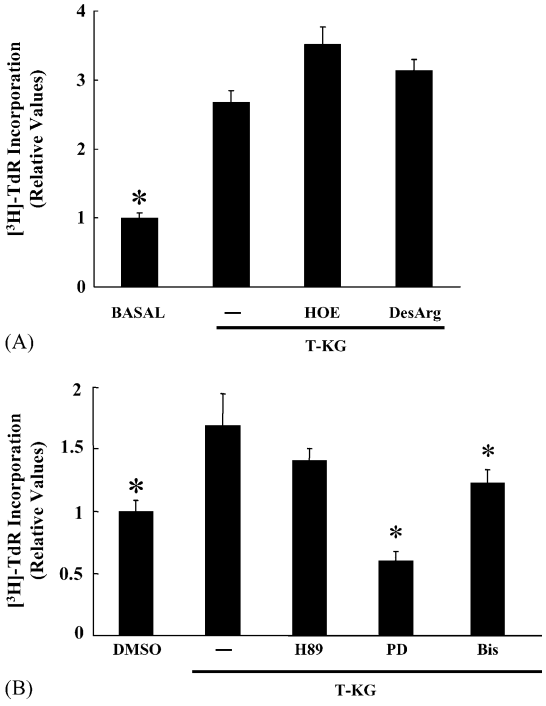

Fig. 4. The effect of T-KG on Balb/c 3T3 fibroblasts does not require kinin receptors, but requires ERK activity. Cells were seeded and serum-starved for 48 h as before. Kinin receptor antagonists (panel A) or signal transduction inhibitors (panel B) were added 30 min before T-KG addition (2 μg/ml). Incubation was then continued for a total of 24 h, and [^{3}H]-TdR incorporation was measured during the last 12 h. All measurements were done in triplicate, and the experiments repeated at least three times. Graphs represent the mean ± S.E.M., and data are presented relative to the controls (without T-KG, and either with (panel B) or without (panel A) DMSO). Asterisks denote significant differences ($P < 0.01$) relative to cells treated with T-KG alone. HOE: HOE-140, DesArg: Des-Arg^9-Leu^8-BK, PD: PD098056, Bis: bis-indoleylmaleimide.

On the other hand, Fig. 4B indicates that the effect of 2 μg/ml T-KG on [^{3}H]-TdR incorporation is completely abrogated in the presence of the MEK inhibitor PD098056, and partially eliminated after inhibition of the PKC pathway (by bis-indoleylmaleimide). In contrast, the stimulatory effect was not significantly affected by an inhibitor of the PKA pathway, H89. These results indicate that entry into S phase by the addition of exogenous T-KG requires activation of the ERK pathway of signal transduction, via a mechanism that does not involve the kinin receptors.

3.5. Exogenous T-KG induces ERK activation and synthesis of cyclin A

The involvement of ERK and entry of the cells into S phase was further confirmed by directly measuring ERK activation and cyclin A synthesis in response to T-KG. Fig. 5A shows that exogenous addition of T-KG results in ERK phosphorylation, with both kinetics and strength comparable to those observed after serum stimulation. Consistent with this result, Balb/c 3T3 fibroblasts started synthesis of cyclin A at approximately 16 h after treatment with 2 μg/ml T-KG (Fig. 5B). Again, this result is both quantitatively and kinetically comparable to serum stimulation by 10% FBS. These observations further confirm the data from [^{3}H]-TdR incorporation experiments, which indicate that exogenous T-KG can induce entry into S phase or DNA synthesis in cultured fibroblasts.

4. Discussion

We have previously reported that expression of T-kininogen is considerably increased in the liver of old rats (Sierra et al., 1989), leading to increased serum levels (Sierra et al., 1992). Our previous work has also shown that T-KG can dramatically inhibit cell proliferation when expressed endogenously in Balb/c 3T3 or L_{TK-} fibroblasts (Torres et al., 2001). This inhibition appears to be the result of decreased ERK activity, as a consequence of stabilization of the phosphatase MKP-1 (Torres et al., 2000, 2001). While the protein has been detected in a variety of tissues (Chao et al., 1988; Damas et al., 1992; Gao et al., 1992; Oza et al., 1990), its mRNA has been observed primarily in hepatocytes. Thus, even though fibroblasts can express T-KG in response to cAMP, prostaglandin E2 and other cytokines (Takano et al., 1995), ectopic expression of the protein in these cells is not physiologically relevant. Fibroblasts are not normally exposed to serum proteins either, but this can happen under pathological conditions where there is either plasma transvasation or a rupture of the endothelial layer. Thus, fibroblasts can be viewed as a valid model for exogenous exposure of cells to circulating T-KG under pathological conditions, and perhaps during aging. Therefore, we decided to further test the possibility that T-KG

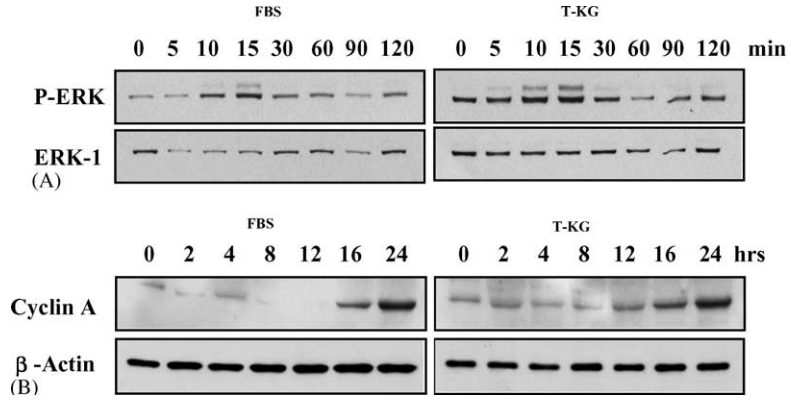

Fig. 5. T-KG induces ERK activation and cyclin A synthesis in Balb/c 3T3 fibroblasts. Cells were seeded and serum-starved for 48 h as before, and then they were stimulated either by 10% BFS or by 2 μg/ml T-KG. (A) ERK phosphorylation (P-ERK) was measured at short times after induction (up to 2 h). (B) Cyclin A accumulation was measured at times up to 24 h. Western blot analysis was performed and representative blots are shown, probed for phosphorylated ERK (P-ERK) and total ERK (ERK-1) (A), as well as cyclin A and β-actin (B).

might inhibit entry into DNA synthesis when added exogenously to these cells.

Our preliminary experiments were surprising, as we observed an induction of [^{3}H]-TdR incorporation, exactly the opposite of what we had expected. Nevertheless, the effects were very reproducible, observed under both quiescent (low serum) and logarithmic growth conditions, and they were concentration-dependent within the sub-physiological range of concentrations tested. Interestingly, we had observed a similar result before, by using conditioned medium from the transfected cells (FS, unpublished data). Due to the untidy nature of these early experiments, they were not pursued further at the time, and instead, purified T-KG was used for all further experiments. Thus, while it is still theoretically possible that an extremely powerful minor contaminant in our preparation could be responsible for the effects we have observed, these experiments suggest that the same molecule can indeed produce both a pro- and an anti-proliferative effect, depending on which side of the plasma membrane it finds itself. One possible explanation for the discrepancy with our previous results is cell drifting. To test this possibility, we repeated our previous transfection studies, using the same batch of cells we were using for the exogenous application experiments. Even though we used a different promoter, these experiments resulted in confirmation of the previous results, whereby ectopically expressed T-KG inhibits [^{3}H]-TdR incorporation. It is possible to argue that we observed the opposite effect with exogenous T-KG because in this case there might be a release of free T-kinin from the precursor, and kinins are known to induce proliferation in other cell types (Dixon and Dennis, 1997; Velarde et al., 1999). Several lines of evidence argue against this possibility. First, using endothelial cells, which are the most active kinin-releasing cells, we have not observed either processing or disappearance of the precursor (measured by Western blot), or appearance of kinins (measured by RIA) (V. Pérez, data not shown). Most definitely, however, at an initial concentration of 2 μg/ml, processing of the available T-KG to completion would only release a maximum of less than 30 nM of T-kinin, a concentration at least 30-fold lower than that shown to induce proliferation of smooth muscle cells (Yang et al., 2003) or keratinocytes (Cheng et al., 2004). Thus, by focusing on a single cell type, we conclude that T-KG exerts a differential effect on the proliferation of Balb/c 3T3 fibroblasts, depending solely on the administration route.

It is likely that the mechanisms involved are very different. Fibroblast proliferation is also inhibited in the presence of cell-penetrating cysteine proteinase inhibitors (Torres et al., 2000). This, together with the observation that ectopic expression of T-KG results in the stabilization of the phosphatase MKP-1, led us to propose that inhibition of proliferation occurs through the cysteine proteinase-inhibitory activity of T-KG (Torres et al., 2001). It is unlikely, however, that a similar mechanism would apply to exogenously added T-KG. First, the physiological effect is the opposite. Second, by using GFP-tagged T-KG, we have not been able to observe any significant internalization of T-KG into cells, including fibroblasts (data not shown). Experiments in lymphocytes, macrophages and endothelial cells have shown specific and saturable binding of FITC-labeled T-KG to sites that appear to be distinct from classical kinin receptors (data not shown). Thus, a more likely alternative is that T-KG might bind to membrane receptors coupled, either directly or indirectly, to activation of the ERK pathway of signal transduction. Binding to surface receptors that activate the ERK pathway would be consistent with the similarities we have observed when comparing T-KG exposure to serum exposure in terms of the kinetics of activation of both ERK and cyclin A expression. Several surface molecules that bind T-KG have been described, including uPAR, cytokeratin 1 and gC1qR (Kusuman et al., 2004), among others. To date, it has not been reported whether or not binding of T-KG to these or other molecules elicits a signal transduction cascade within the cell.

The apparent proliferative effect of exogenous T-KG on fibroblasts is not unique to this cell type. Recent experiments (V. Pérez et al., in preparation) have indicated that a similar effect is observed in a variety of endothelial cells and cell lines. Endothelial cells are particularly rich in kinin receptors, and our data indicate that in this case, the effect does require their activity, which also results in activation of both ERK and PI3K, leading to increased [^{3}H]-TdR incorporation. However, the mode of administration is not the only variable that determines whether a proliferative or quiescence response is induced. Indeed, even when administered exogenously, T-KG inhibits both basal proliferation and the proliferative response given by Con A or PHA, both in Jurkat cells and primary rat splenocytes (Acuña-Castillo et al., in preparation). Both these events require ERK activity, and T-KG does not affect the proliferative response of Jurkat cells to IL-2, a process that does not require ERK activity. Thus, we conclude that T-KG can have pleiotropic effects on cell proliferation, depending both on the mode of administration and the cell type under study.

It is possible that the differences observed among different cell types could be related to the number and type of receptors they express on their surface. The effect of T-KG is independent of kinin receptors in fibroblasts and Jurkat cells, but it is dependent on them in the case of endothelial cells. Western blot analysis indicates that the most abundant (and constitutive) kinin receptor, B2, is present on both endothelial cells and fibroblasts, both of which respond by an increased proliferation, but B2 receptors were not detected in Jurkat cells, which respond by inhibition. Certainly, this is not the entire story, because these receptors were shown not to be required for the effect in fibroblasts. Thus, it is likely that other T-KG binding surface molecules might modulate the final responsiveness of the cell.

It is noteworthy that the effects we have observed occur at sub-physiological concentrations of T-KG, and therefore it is likely that some mechanism must exist for dampening these effects in vivo. Indeed, we have observed that in the serum from young rats, most of the available T-KG exists in the form of complexes with other proteins, and we speculate that these complexes might limit the bioavailability of T-KG. This would limit the physiological relevance of our in vitro findings. However, with increasing age, the increase in T-KG expression leads to the appearance of free T-KG (data not shown). Thus, while cell proliferation might not be significantly affected by T-KG in young animals, it is likely that free circulating T-KG might affect the proliferative homeostasis of old rats.

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