Constitutive degradation of IκBα in human T lymphocytes is mediated by calpain
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Abstract

Background: Activation-induced induction of transcription factor NFκB in T lymphocytes is regulated by its inhibitor IκBα. NFκB activation has been demonstrated to occur either by phosphorylation on serine residues 32 and 36 of the inhibitor, IκBα, followed by ubiquitination and degradation of the inhibitor by the 26S proteasome, or by a proteasome-independent mechanism involving tyrosine phosphorylation, but not degradation. However, the mechanism underlying constitutive regulation of the levels of the inhibitor, IκB, in primary human T lymphocytes, remains to be fully delineated.

Results: We demonstrate here, the involvement of a proteasome-independent pathway for constitutive regulation of IκBα levels in primary human T lymphocytes. Pretreatment with a cell permeable calpain inhibitor, E64D, but not with a proteasome specific inhibitor, lactacystin, blocks stimulus-independent IκBα degradation in primary human T cells. However, E64D pre-treatment fails to impact on IκBα levels following stimulation with either TNFα or pervanadate. Other isoforms of the inhibitor, IκBβ, and IκBγ, appear not to be subject to a similar ligand-independent regulation. Unlike the previously reported decline in ligand-induced degradation of IκBα in T cells from the elderly, constitutive degradation does not exhibit an age-associated decline, demonstrating proteasome-independent regulation of the activity.

Conclusion: Our studies support a role for an E64D sensitive protease in regulating constitutive levels of IκBα in T cells, independent of the involvement of the 26S proteasome, and suggests a biological role for constitutive degradation of IκBα in T cells.

Background

Transcription factor NFκB exists as homo-or-heterodimeric complexes, consisting of the Rel family of proteins [1]. These dimers operate as transcriptional regulators essential for a variety of cellular processes ranging from cell cycle progression to immune response gene induction [2]. In human T lymphocytes, like most other cells, NFκB exists in the cytoplasm coupled to its inhibitor IκBα or IκBβ, predominant members of IκB family of proteins [3]. A high affinity for RelA and c-Rel molecules enables these inhibitory proteins to associate with and thus restrict nuclear localization of the NFκB molecules. Most stimuli responsible for NFκB induction have been demonstrated to either invoke serine phosphorylation of the
inhibitory proteins followed by ubiquitination and degradation via the 26S proteasome pathway, or involve the activation of tyrosine phosphorylation, as in the case of oxidative stress mediated stimuli, which is independent of proteasomal degradation mechanism [4,5]. While stimulation-induced modification of IκB has been studied extensively, little is known about the constitutive regulation of IκB protein in T cells under resting conditions.

Recent studies in B cell lines have demonstrated that IκBα, but not IκBβ, is constitutively degraded and is important for the induction of constitutive NFκB activity [6,7]. These studies indicate that constitutive degradation of IκB is mediated by a proteasome independent pathway. Studies also suggest that a calcium-dependent protease, calpain, may be important in regulating levels of IκBα [8-10]. These studies prompted us to investigate whether a similar regulation of "constitutive" i.e., stimulus-independent levels of IκBα occurs in primary T lymphocytes. Unlike B cells, NFκB induction and IκB regulation reported in T cells is clearly mediated by exogenous activating stimuli, with little or no constitutive nuclear NFκB present under basal condition.

Further, as NFκB regulation is significantly altered during aging in human T cells, we examined whether abnormal constitutive regulation may underlie lowered activation-mediated induction. Employing primary T cells obtained from human donors, we evaluated whether aging affects the regulation of constitutive levels of IκBα. We hypothe-
sized that, as constitutive levels of IkBα were relatively unaffected by age in primary T cells, this may reflect minimal effect of age on calpain activity in T cells. We now report that E64D sensitive protease, calpain, is indeed responsible for regulating constitutive levels of IkBα, but not IkBβ or IkBγ, in human T cells. Further, calcium-ionophore mediated increase in calpain activity induced in T cells from young donors showed consistently higher activity at early time points after activation, when compared to the elderly. However, total calpain activity measured at the end of 60 minutes demonstrated no significant modulation based on the age of the donor. Thus, while the kinetics of calpain activation appears to be altered in T cells from the elderly, cumulative activity over a period of time remains unaffected. Additionally, we demonstrate that aging does not significantly affect stimulus-independent degradation of IkBα mediated by calpain, demonstrating proteasome-independent regulation. Thus, the calpain system is involved in the constitutive regulation of IkBα, and hence the NFκB signaling pathway, under resting conditions, in primary human T lymphocytes.

Results
Treatment with a cell permeable cysteine protease inhibitor, E64D, inhibits constitutive degradation of IkBα but not IkBβ or IkBγ in T lymphocytes from young and elderly donors
We examined the effect of a cell permeable cysteine protease inhibitor, E64D, on the basal levels of IkBα, IkBβ and IkBγ, inhibitors of the ubiquitous transcription factor NFκB. Employing primary T cells and western blotting using antibody specific to the inhibitor isoforms, we now demonstrate that constitutive levels of IkBα, but not IkBβ or IkBγ are modulated by pretreatment with E64D. Results presented in fig. 1, demonstrate that pretreatment with E64D significantly inhibits constitutive degradation of IkBα.
IκBα with little or no effect on either IκBβ (fig. 2) or IκBγ (fig. 3). Levels of IκBα before treatment with E64D are significantly lower, than that observed following treatment, indicating inhibition of degradation.

As IκBα degradation induced by exogenous signals has been reported to be differentially regulated during aging [8,9], we next assessed the effect of treatment with E64D on IκB-α in T cells obtained from young and elderly donors. Results presented in fig. 1, demonstrate that, irrespective of the age of the T cell donor, E64D pretreatment significantly protected IκBα levels from constitutive degradation, and had little impact on isoforms, IκBβ (fig. 2) and IκBγ (fig. 3). Thus, aging does not influence cysteine protease-sensitive constitutive degradation of IκBα in T cells.

Figure 5
Effect of E64D on stimulus-dependent modification of IκBα (Integrated Densities). T cells obtained from young and elderly donors were either left untreated or treated with E64D (50 μM) for 45 min. At the end of incubation cells were stimulated with 100 μM pervanadate or TNFα (10 ng/ml) for 10, 20 or 30 min. Cell lysates were prepared and resolved by SDS-PAGE, using equal amounts of protein (30 μg/lane). Resolved proteins were transferred to nitrocellulose membrane and Western blotted with antibody to IκBα and detected using ECL. Mean integrated densities of IκBα specific bands obtained from a minimum of 4 donor pairs were used to determine fold change when compared to their respective controls and are presented as Mean (fold-change) ± S.D. [TNF-α (A); Pervanadate (B)].
Inhibition of cysteine protease activity by E64D does not affect TNFα-induced degradation of IκBα in T cells

Treatment of T cells by activating stimuli such as, anti-CD3 or TNFα, have been demonstrated to induce transcription factor NFκB activation by a signal-induced, proteasome-mediated degradation of the inhibitor IκBα. To determine whether such signal-induced degradation of IκBα was subjected to regulation by cysteine proteases, we next examined the role of E64D on activation-induced levels of IκBα. Results depicted in fig. 4 and 5A, clearly demonstrate that activation of T cells with TNFα induces degradation of IκB-α, irrespective of treatment with E64D. This indicates that E64D sensitive protease does not modulate or impact on activation-induced, proteasome-dependent degradation of IκBα. It should be noted that a slower mobility IκBα band appears at later time-points (20 and 30 min.) in TNF activated cells from the elderly. We believe that this represents modified IκBα. Future experiments will determine the precise nature of this modification. As proteasome dependent degradation of IκBα is clearly differentially regulated in T cells from young and elderly donors, we next examined, whether E64D mediated inhibition of constitutive levels impact differentially on the induced degradation of IκBα in T cells from the elderly. Results presented in fig. 5A, demonstrate that pre-treatment with E64D failed to influence activation-induced degradation of IκBα. Thus, irrespective of the age of the donor, treatment with E64D failed to modulate activation-induced degradation of IκBα.

Pretreatment with E64D does not interfere with activation-induced modification mediated by pervanadate treatment in T cells

As a next step in the analyses, we examined the effect of pretreatment with E64D on modes of activation that does not involve signal-induced degradation of IκBα, such as those mediated by pervanadate. Similar to the observation with TNFα, pretreatment with E64D failed to impact on activation-induced IκBα modification in cells pre-treated with pervanadate, irrespective of the age of the donor (fig. 4 and 5B). It is important to note that IκBα in cells treated with pervanadate clearly demonstrate a slower mobility band representing tyrosine-phosphorylated IκBα. Thus, irrespective of the activation stimuli (TNFa or Pervanadate), E64D appeared not to impact on
the activation-induced modulation of IκBα. NFκB dependent luciferase activity was also assayed following pervanadate treatment, in the presence or absence of E64D. In keeping with the data obtained with IκBα, E64D pretreatment, failed to impact on NFκB dependent luciferase activity, (data not shown).

**Treatment of T cells with proteasome inhibitor, Lactacystin, does not influence constitutive IκB-α levels, unlike that mediated by E64D**

As IκB-α is constitutively degraded by E64D sensitive cysteine protease, we next assessed whether treatment with a proteasome inhibitor also interfered with this basal degradation. Results presented in fig. 6A, clearly demonstrate that pretreatment with lactacystin, a proteasome specific inhibitor, failed to influence basal levels of IκBα. Thus suggesting that basal or constitutive regulation of IκB-α is not dependent on the proteasome. To ensure that lactacystin did inhibit proteasome at the dose employed, i.e. positive control, T cells from young and elderly donors were either pretreated with lactacystin or left untreated. These cells were then subjected to treatment with TNFa. As seen in fig. 6B, TNFa treatment in the young induced degradation of IκBα, when compared to untreated controls. Pretreatment with lactacystin and then TNFa (L+T), inhibited TNFa mediated degradation. As reported previously, lactacystin is only minimally effective in T cells from the elderly.

**Treatment of T cell lysates with purified calpain, mimics constitutive degradation of IκBα**

As E64D pretreatment specifically inhibited basal degradation of IκBα, implicating a role for cysteine proteases such as calpain in regulating the constitutive levels of IκBα, we tested for a direct role for calpain by treating cytosolic lysates obtained from T cells with a purified preparation of calpain in an in vitro assay. T cell lysates equalized for protein from young donors were pooled and subjected to lysis in the presence of purified calpain, as indicated. Degradation of IκBα was determined by measuring the detectable levels of IκBα by SDS-PAGE and western blotting using antibody to IκBα. Results presented in fig. 7, show that upon exposure to 0.01 U of calpain, lysates from T cells demonstrate lowered levels of IκBα, indicating calpain-mediated degradation, which is inhibited by pretreatment with E64D.

**Kinetics of induction of calpain activity following calcium ionophore treatment, is modulated by age, but has no impact on overall effective calpain activity in T cells**

To assess the effect of age on calpain activity, we obtained T cells from young and elderly healthy volunteers and examined them for endogenous calpain activity. As demonstrated in fig. 8, calpain specific protease hydrolyzing activity appeared to be slightly higher at time 0 in T cells from young donors; however, calcium ionophore induced increase in calpain activity was not significantly different between T cells from young and elderly donors. In fact, total hydrolyzing activity measured at the end of 60 minutes was not statistically different between cells obtained from the two age groups. Therefore, effective calpain activity remained unaffected by age of the T cell donor.

**Discussion**

Calpain system has been demonstrated to be the main protease involved in constitutive degradation of IκBα [7-9]. Delineation of the exact region of IκBα necessary for degradation by calpain resulted in the identification of the C-terminal 39 amino acid sequence containing the PEST sequence to be critical for degradation in vitro by Shumway and Miyamoto [6]. However, their studies, demonstrated that calpain was not responsible for the degradation of IκBα in primary B cells [6]. Unlike B cells, that express constitutive nuclear NFκB, significant nuclear expression of NFκB in T cells occurs predominantly following stimulus-induced activation. Few studies, to date, have delved into constitutive regulation of NFκB in resting T cells. In our current studies, employing E64D, we demonstrate specificity of constitutive degradation of IκBα mediated by calpain in human primary T lymphocytes. The inhibition of this degradation in the presence of E64D, a cell permeable, cysteine protease inhibitor, supports the potential involvement of calpain activity in this process. Given the central role of NFκB in cell survival and signaling [11], constitutive degradation of the inhibitor IκBα is vital in understanding steady state kinetics of T cell regulation in the context of immune activation.

Our studies also demonstrate that degradation of IκBα under resting condition is refractory to proteasome inhibitor, Lactacystin, but not to calpain inhibitor, E64D. Therefore, unlike that reported for activation-induced degradation [12], constitutive levels of IκBα appear not to be subject to proteosomal regulation. This is particularly important given that our previous findings clearly showed that inducible degradation of IκBα is subject to an "age-effect" due to the inhibitory action of aging on proteasome-associated proteolytic activity [13,14].

Calpain-dependent degradation of IκBα has been demonstrated to occur in other cell types, which are refractory to proteasome activity [11]. Thus, it appears, that the degradation of IκBα can occur through two mutually exclusive pathways, dependent on the state of the cells, i.e., resting versus activated. Calpain system plays a role in constitutive, but not induced IκBα degradation, while proteasome degradation dictates induced levels in T cells [6,12]. Calpain activity has been demonstrated to be involved in the degradation of IκBα under certain conditions of viral infection [8]. It is therefore likely that this ability of con-
constitutive degradation may be exploited by certain patho-
gens.

Unlike the most predominant inhibitor IκBα: IκBβ and
IκBγ isoforms, appear not to be susceptible to this calpain-
mediated degradation. Recent elegant experiments by
Miyamoto et al implicate similar degradation kinetics for
IκBα isoform in B cell lines [6]. Drawing upon the signif-
icance of such degradation events in the constitutive
induction of NFκB in B cells, the role for constitutive regu-
lation of NFκB by the calpain pathway in primary T cells
was examined here. Results from these experiments
clearly provide a biological basis for stimulus-independent
degradation and its importance in the maintenance of
NFκB in cell survival, which is not evident, unless chal-
lenged by stimuli capable of inducing apoptosis (data not
shown).

Our studies on the effect of advancing age on constitutive
degradation of IκBα, clearly implicate absence of any
effect of donor age on the maintenance of E64D protease
sensitive/calpain activity responsible for this degradation.
Experiments conducted to determine the impact of aging
on calpain activity clearly indicate that the effective activ-
ity is unaltered during aging. This is also reflected in the
levels of IκBα in resting T cells from young and elderly
donors, which are unaffected by age. Thus, despite loss in
proteasome activity accompanying aging, calpain-medi-
dated degradation of IκBα remains unaltered, demonstrat-
ing little or no role for the proteasomal regulation in
calpain-mediated pathway that regulates IκBα levels. This
observation is in keeping with earlier reports from our
laboratory that demonstrated minimal effect of age on
overall cellular proteolytic activity, especially, T cell chy-
motryptic activity [15]. It is also interesting to note that
reports on calpain activity as a function of advancing age
have been conflicting, with some demonstrating increased activity, [16,17] and others, decreased activity
[18,19], however, these studies either used other cell
types, employed exogenous substrates or cell lysates for
the evaluation of the activity. Using a fluorogenic model
substrate that is cell permeable, we now demonstrate that,
ionomycin-inducible specific calpain activity, inhabitable
by E64D, is unaffected; however, proteolytic activity
observed in T cell lysates appeared to follow different
kinetics in cells from the young than those observed in the
elderly. Importantly, while 90% of the activity in cells
from the young was clearly inhabitable by treatment with
E64D, only 50% of the activity was inhabitable in cells
from the elderly (data not shown).

While constitutive degradation of IκBα is clearly regulated
by E64D sensitive calpain in T cells, activation-induced
degradation, appears unaffected by pretreatment with
E64D. Similarly, while activation-induced degradation of
IκBα is sensitive to proteasome inhibition, constitutive
degradation is unaffected by pretreatment with lactacys-
tin, a proteasome inhibitor. Clearly, susceptibility of IκBα
to degradation is not only dependent on the state of acti-
vation but also on the specificity of the protease. The
physiologic significance of the degradation of the inhibi-
tor clearly dictates induction of NFκB levels, and thus
anti-apoptotic or survival ability, under uninduced condi-
tions. Thus, regulation of IκBα levels in basal state of a cell
is crucial and sets the stage for activation-induced survival
signals. These results also indicate that the calpain path-
way works independently of phosphorylation, since nei-
ther TNF nor pervanadate that induce serine and tyrosine
phosphorylation, respectively, were affected by the inhibi-
tor. Further, while proteasome dependent activation-
induced degradation pathway, as well as proteasome
pathway has been demonstrated to be compromised in T
cells during aging, calpain activity clearly appears to be
still functional, and is minimally affected by advancing
age.

Conclusion
In summary, we have demonstrated that basal levels of
IκBα, but not IκBβ or IκBγ are subject to regulation by
E64D sensitive protease, and can be mimicked by pre-
treatment with calpain. The regulation of IκBα levels by
cysteine protease appears to have no effect on activation-
induced IκBα or on other isoforms of IκB, irrespective of
the stimuli employed. Additionally, it appears that inter-
ference with this decrease in basal degradation of IκBα
does not impact on cell survival under resting conditions.

Methods
Materials
Fluorochrome labeled anti-CD3, and FITC- and PE-
labeled isotype controls were obtained from Sigma Chemical
Co. (St. Louis, MO). Anti IgG coupled to horseradish
peroxidase was obtained from BD-Transduction Labora-
tories (Lexington, KY). All other antibodies were from
Santa Cruz Biotech (Carlsbad, CA). Enhanced Chemi-
luminescence reagents were from Amersham (Arlington
Heights, IL). All fine chemicals unless otherwise men-
tioned were obtained from Sigma Chemical Company,
(St. Louis, MO.). Electrophoresis supplies and Molecular
weight standards were from BioRad (Richmond, CA.).
E64D and lactacystin were from Calbiochem (CA).
Substrate for Calpain was from Molecular probes, (Eugene,
OR).

Human subjects
Blood was obtained from healthy individuals living in the
community. Young donors were between 21 and 30 years
and old donors were between 65 and 85 years of age. A
minimum of at least four donor pairs were used in each
experiment. Both young and elderly donors were in good
physical and mental health, had no apparent illness as suggested by an elaborate screening history and were not on any medication directly impacting the immune system during the course of this study.

**T Lymphocyte Isolation**

Peripheral blood was obtained and T cells were purified and maintained in RPMI 1640 culture medium as previously described (20). Magnetic sorting by negative selection was used to isolate CD3+ T cells. Purity of the isolated T cells was determined by flow cytometry using anti-CD3 conjugated to FITC. Populations were 90–95% pure. Treatment of T cells (20 × 10⁶ cells/ml) with pervanadate (100 µM, freshly prepared before use) was carried out for indicated times at 37°C, before cell lysates were prepared. For experiments involving the use of inhibitor, cells were treated with E64D at 50 µM for 45 minutes.

**Western bloting**

Cytosolic extracts for Western blotting were prepared by homogenization of cells in lysis buffer (1 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, and 1 mM sodium orthovanadate, and 0.5% NP-40) (20). The following reagents were added to all buffers prior to their use: 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor. Protein content of cytosolic extracts was determined using BioRad protein assay. Cell lysates equalized for protein (40 µg) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, immuno-blotted with specific antibody/s, and detected using anti-IgG coupled to horseradish peroxidase followed by Enhanced Chemi-luminescence (ECL). Where possible samples from young and elderly donors were resolved on the same gel, but in experiments where different treatments were analyzed, samples from young and the elderly were resolved on different gels, but were run simultaneously, to avoid inter and intra experimental variability. Resolution of samples on different gels did not influence the outcome of the results.

**Calpain activity assays**

Using a cell permeable fluorescent substrate Boc-Met-AMC, calpain activity within live cells was measured using a spectrofluorometer. Fluorescence was measured in cell suspension (2.5 × 10⁶ cells/ml) following the addition of fluorophore, using the LS-50 model, Perkin-Elmer Spectrofluorometer. The fluorometer was equipped with a magnetic stirrer and warmed with recirculating water at 37°C using a pump. Fluorescence was measured using excitation and emission wavelengths of 380 and 460 nm, respectively. Values were obtained using a time drive mode, for up to 60 minutes.

**Statistical analyses**

Data were analyzed using student’s t-test. Differences were considered significant, if p < 0.05.

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