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Bio-effectiveness of Tat-catalase conjugate: a potential tool for the identification of H_2O_2-dependent cellular signal transduction pathways☆

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Abstract

Reactive oxygen species such as hydrogen peroxide (H_2O_2) have taken center stage as bona fide second messengers in various signaling pathways. Here, we report the synthesis, metabolic fate, and effectiveness in modulating such pathways of a Tat-catalase conjugate. Incubation of L2 cells with Tat-catalase greatly increased cell-associated enzymatic activity, reaching close to a plateau by 30 min. The cell-associated catalase activity and antibody-detectable Tat-derivatives declined over time after changing medium, although still remaining at significantly higher levels than baseline even at 4 h. While most cell-associated Tat-catalase was apparently tightly attached to the cell surface, a small fraction entered the cells as the proteasome inhibitor MG-132 slightly prevented the disappearance of the enzyme. Tat-catalase, either membrane-bound or intracellular, but not native catalase, inhibited serum-induced Elk phosphorylation and anisomycin- and/or MG-132-induced ERK phosphorylation, suggesting the involvement of H_2O_2. Thus, Tat-catalase should be a useful tool to dissect H_2O_2-dependent events in signaling pathways.

Keywords: Catalase; Transduction; MAP kinase; Redox signaling; Hydrogen peroxide; Tat; PTD

Certain viral proteins such as HIV Tat and HSV VP-22 can enter cells across the plasma membrane by a poorly understood mechanism. A stretch of basic amino acids, branded as the protein transduction domain (PTD), has been credited with providing the ability of these proteins to accomplish this feat. The minimum PTD segment of the HIV Tat protein corresponds to residues 49–57 (RKKRRQRRR). PTD-dependent transduction has been characterized as rapid, with uptake occurring within minutes of exposure and energy-independent (transduction even at 4 °C) [1]. These characteristics led to the conclusion that mechanisms other than endocytosis were involved in this process, which also appears to be receptor-independent as recent studies with sequence inversion or with polyarginine [2,3] and polylysine peptides [4] showed that the positively charged nature of these peptides, rather than the sequence itself, was important for transduction.

The ability to deliver molecules into cells has generated excitement and many exogenous molecules have been linked to a PTD or a polymer of basic amino acids, either chemically or in the form of fusion proteins. The cargo molecules that were reportedly delivered into cells, as assessed mostly by fluorescence

☆ *Abbreviations:* PTD, protein transduction domain; mica, maleimidocaproic amide; LA, low enzyme activity; HA, high enzyme activity; NaPi, sodium phosphate buffer; MAPK, mitogen-activated protein kinase; ERK, extracellular-regulated kinase; JNK, c-Jun N-terminal kinase.

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microscopic examination of fixed cells, were diverse in nature and included bioactive peptides [5], a wide variety of high molecular weight proteins including β-galactosidase [6,10], and even DNA packaged phage [7] and magnetic beads [8,9]. The most convincing evidence for cell delivery came from studies in mice intraperitoneally injected with a Tat-PTD-β-galactosidase fusion protein, which was quickly delivered to all tissues, including the brain [10]. PTD fusion proteins produced bacterially from expression vectors exhibited higher delivery when first denatured, apparently followed by refolding in the cytoplasm (reviewed in [11]), whereas PTD-protein conjugates coupled using cross-linking reagents did not necessarily require denaturation [2,6]. Recent studies have argued that the rapid non-endocytotic transduction and especially the subsequent nuclear localization of Tat-derivatives was artifactual [12,13] and have suggested that endocytosis may actually be involved [14]. Nevertheless, several cargo molecules have now been shown to exert the expected biological effects [4,5,15–17], indicating that internalization must have occurred.

As hydrogen peroxide (H_2O_2) has gained credence as a key second messenger in a wide variety of circumstances ranging from proliferation to apoptosis (reviewed in [18,19]), catalase transduction by Tat PTD would be a useful approach for the study of redox cell signaling. Jin et al. [20] previously demonstrated that a bacterially expressed catalase fusion protein with Tat PTD or polyarginine transduced into cells, rendering them resistant to oxidative stress and penetrated mice skin. Here, we have evaluated the ability of a Tat PTD-catalase conjugate that was chemically coupled using a cross-linking reagent to enter cells and tested its effectiveness using the activation of the mitogen-activated protein kinase (MAPK) pathways as a model assay system. The data show that exposure of L2 cells to Tat-catalase dramatically increased cell-associated catalase activity, inhibiting serum-induced Elk phosphorylation and anisomycin- and/or MG-132-induced ERK phosphorylation. Thus, the Tat-catalase can be a useful research tool to dissect the signal transduction pathways involving H_2O_2-dependent events.

Materials and methods

Cells and reagents. Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO). Rat lung L2 cells were purchased from the American Type Culture Collection (ATCC) and were cultured in F-12K medium (Gibco-BRL) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified atmosphere containing 5% CO_2 at 37 °C.

A monoclonal antibody to the Tat protein transduction domain (PTD; RKKRRQRRRPPQG) was generated at the monoclonal antibody facility at UAB, using a Tat-PTD-keyhole limpet hemocyanin conjugate as antigen and Tat-PTD-BSA conjugate as an immunosorbent for the screening.

Synthesis of Tat-catalase. Tat PTD peptide, GRKKRRQRRRP PQG, was synthesized according to the automated base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) strategy from each protected amino acid (AnaSpec, San Jose, CA) and Fmoc-Gly-Wang resin (Perkin–Elmer, Norwalk CT) on the Applied Biosystems model 431 peptide synthesizer. Incorporation of 6-maleimidocaproic amide (mica) to the N-terminal Gly residue of the peptide was achieved on the resin through reaction with HOBt-6-maleimidocaproic acid ester (Fluka, Milwaukee, WI). The peptides were cleaved off the resin by treatment with 90% trifluoroacetic acid, 5% thioanisole, and 5% H_2O for 3 h. The peptides were subsequently filtered from resin, precipitated using *tert*-butyl methyl ether, purified by reverse-phase high-performance liquid chromatography, and then characterized by Lasermat 2000 matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Finnigan, San Jose, CA).

The mica-Tat PTD peptide was incubated with bovine liver catalase (2.5 mg/ml) at a 10:1 molar ratio of peptide to monomer enzyme in 0.1 M NaPi, pH 7.4, for 48 h at 4 °C. Almost all the catalase was modified with Tat peptide under these conditions. The Tat-conjugate was separated from unconjugated Tat peptide by Sephadex G50 gel filtration ($3.5\,cm^2 \times 10\,cm$) using PBS as solvent. The Tat-catalase elution fractions were pooled and concentrated by ultrafiltration (Centricon YM-10; Millipore, Bedford, MA) and the purity was assessed by separation on 10–20% Tris–Tricine or 10% Tris–Glycine SDS–PAGE and Coomassie staining. Although the unconjugated mica-Tat peptide was completely undetectable by these methods (Fig. 1A), minute amounts of contaminating peptides were unavoidable in the final pool, possibly due to some interaction between the positively charge peptide and the modified enzyme. This contaminating peptide could form a conjugate with proteins in cell lysates, artifactually generating anti-Tat-antibody-detectable molecules (see Figs. 2B and D). For this reason, the percentage of active maleimide moiety of the unconjugated mica-Tat peptides was minimized in a subsequent preparation lot by prior quenching of the crude conjugates with Cys

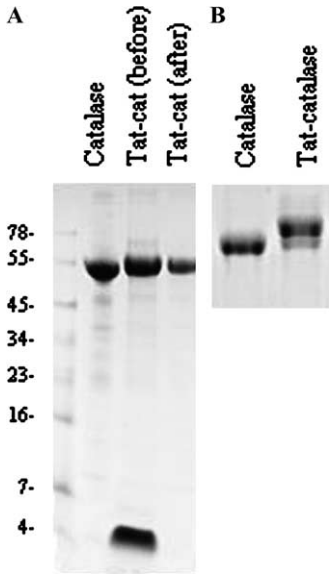

Fig. 1. SDS–PAGE analysis of Tat-catalase conjugate Mica-Tat peptide was coupled with catalase and the resultant Tat-catalase conjugate was separated from the unconjugated peptide by gel filtration, as described in Materials and methods. The crude and gel-filtered fraction pool of HA Tat-catalase was analyzed with Coomassie staining following resolution by 10–20% Tris–Tricine SDS–PAGE (A, ~ 5 μg/lane) or 10% Tris–Glycine SDS–PAGE (B, 2 μg/lane). The numbers on the left side of the gel (A) indicate molecular weight in kilodaltons.

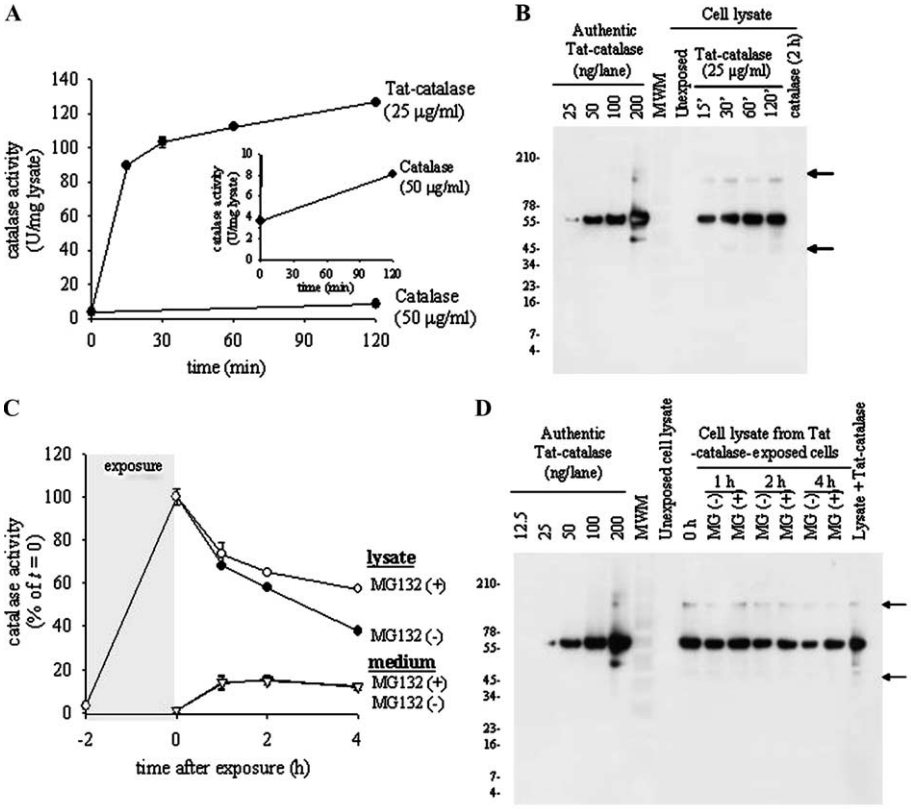

Fig. 2. Time course of transduction and the subsequent metabolism of Tat-catalase in L2 cells. (A) Time course of transduction: L2 cells were incubated with catalase (50 μg/ml) or LA Tat-catalase (25 μg/ml) in culture medium for the indicated periods and catalase activities were measured. The inset graph shows the magnified part of catalase activity in cells exposed to control catalase. Values are means ± range of two samples. (B) Immunochemical detection of Tat-catalase in the exposed cells. Aliquots from the same cell lysates as in (A) (1.25 μg/lane) were subjected to immunoblotting with anti-Tat antibody. The arrows indicate Tat-derivatives from cellular proteins due to minute contamination by the mica-Tat peptide in the enzyme fraction (see legend to D). The left part of the blot is an internal standard made by serially diluting authentic Tat-catalase. The numbers on the left side of the blot indicate molecular weight in kilodaltons. (C) Change in catalase activity following the end of exposure in the absence or presence of the proteasome inhibitor MG-132. Following the 2 h incubation with 25 μg/ml LA Tat-catalase ($t = 0$), cells were washed with PBS and incubated with fresh medium with or without MG-132 (20 μM) for the indicated times, and the catalase activity in the cells and the media was measured. Catalase activities were normalized to that for $t = 0$ (140 ± 8 U/mg lysate). Values are means ± range of two samples. (D) Change in Tat-catalase molecule following the end of exposure in the presence or absence of the proteasome inhibitor MG-132. The aliquots of cell lysate of (C) (1.25 μg/lane) were subjected to immunoblotting with anti-Tat antibody. The left part of the blot is an internal standard made by serially diluting authentic Tat-catalase. The arrows indicate Tat-derivatives generated from cellular proteins due to minute contamination by mica-Tat in the enzyme fraction that could be most easily observed by mixing Tat-catalase with cell lysate in vitro (right most lane). The numbers on the left side of the blot indicate molecular weight in kilodaltons. Note that the presence of MG-132 partially prevented the decrease in the amount of Tat-catalase.

(10 times molar excess of the original mica-Tat peptide) for 24 h at 4 °C before gel filtration. The quenching, which significantly, although not completely, inactivated the maleimide moiety involved in the final products, entailed a loss of two-thirds of the enzyme activity. Thus, two Tat-catalase lots with different enzyme activities were obtained and used in this study, hereby referred to as high enzyme activity Tat-catalase (HA Tat-catalase) and low enzyme activity Tat-catalase (LA Tat-catalase) (see Results). Catalase, similarly gel-filtered, was used as control for each experiment.

Tat-catalase transduction. L2 cells in 12-well plates ($\sim 1.2 \times 10^5$/well) were incubated with 500 μl Tat-catalase (25 μg/ml) in the culture medium for defined times and washed with PBS (2 ml × 3 times). For subsequent metabolic chasing studies, the 2 h-exposed cells were washed with PBS as above, incubated with fresh medium in the absence or presence of the proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-leucinal, 20 μM; Sigma), and washed with PBS at the end of the incubation. Cells were lysed in 150 μl of 0.1% Triton X-100/NaPi, pH 7.4, on ice, and centrifuged at 10,000*g* for 15 min at 4 °C to obtain the supernatant. The great difference in catalase activity in lysates between Tat-catalase-treated cells and untreated cells necessitated using different amounts of lysates for the subsequent enzyme assay. Therefore, lysates from 3 wells of untreated or catalase-treated cells were combined and used as one sample, whereas lysates from a single well of Tat-catalase-treated cells were used individually. Protein concentration of the lysates was determined by the Bradford method (BioRad) with bovine serum albumin as a standard.

Catalase activity in the lysate was measured spectrophotometrically using 10 mM H_2O_2 as a substrate ($\varepsilon_{240} = 39.4\,M^{-1}\,cm^{-1}$). Catalase activity in the culture medium, which itself contains a potent non-enzymatic H_2O_2 scavenging activity, was evaluated as the rate of sodium azide-inhibitable H_2O_2 decomposition. Sodium azide (50 μM) completely inhibited catalase activity but had no effect on the H_2O_2 scavenging activity of the medium itself (data not shown).

The aliquots of the lysates (1.25 μg) were resolved by 10–20% Tris–Tricine SDS–PAGE, transferred onto PVDF membrane (Immobilon P; Millipore), and subjected to immunoblotting analysis using anti-Tat PTD monoclonal antibody (5000× dilution). The bound primary

antibody was detected by horseradish peroxidase-labeled secondary antibody, followed by the enhanced chemiluminescence visualization (ECL; Amersham) according to the manufacturer's instructions.

Activation of the map kinase pathways. L2 cells in 12-well plates (1.2×10^5/well) were serum-starved for 17 h (10–0.5%). Cells were preincubated with 500 μl of either catalase (50 μg/ml) or Tat-catalase (10 or 50 μg/ml), together with or without MG-132 (20 μM), in 0.5% serum containing medium for 2.5 h, and then stimulated for 20 min with serum (20%) or anisomycin (10 μg/ml) by adding 1/4 volume of each 5-fold final concentration solution (catalase and Tat-catalase were therefore present throughout stimulation). Cells were washed with PBS and immediately lysed with 80 μl of 1× SDS–PAGE sample buffer, followed by boiling for 3 min. Samples were then resolved by 10% Tris–Glycine SDS–PAGE and analyzed by immunoblotting as described above using at the indicated dilutions antibodies to the phosphorylated forms of ERK (p-ERK; E-4: 1000× dilution), Elk (p-Elk; B-4: 1000× dilution), and c-Jun (p-c-Jun; KM-1: 1000× dilution) or an antibody against the ERK 1 protein (K-23: 4000× dilution), all from Santa Cruz Biotechnology (Santa Cruz, CA).

Results and discussion

Synthesis of the conjugate was initially carried out by coupling succinimide ester-incorporated Tat peptides to lysine residues in catalase. However, the N-linked conjugation caused aggregation of the modified enzyme as well as loss of enzyme activity. Therefore, we conjugated a maleimide-modified Tat peptide to cysteine residues in catalase. This S-linked conjugation caused neither aggregation nor severe loss of the enzyme activity. The enzyme activities of the final Tat-catalase preparations obtained with and without quenching with Cys at the end of coupling (see Materials and methods) were 15,200 U/mg (HA Tat-catalase) and 5000 U/mg (LA Tat-catalase) whereas that for the control catalase was 21,100 U/mg.

Fig. 1A shows the purity of HA Tat-catalase by SDS–PAGE and Coomassie staining before and after Sephadex G50 gel filtration. Unconjugated Tat-peptide was not detected in the final Tat-catalase fraction under these conditions. Higher resolution gel (10%) showed that native catalase was almost completely modified to the higher molecular weight Tat-catalase by this method (Fig. 1B). Nearly identical results were obtained with LA Tat-catalase (data not shown).

Kinetics of "transduction" and subsequent metabolism of Tat-catalase

L2 cells were incubated with Tat-catalase for various time periods and cell-associated catalase activity was determined. As shown in Fig. 2A, within 30 min of exposure to LA Tat-catalase at 25 μg/ml, the catalase activity associated with the exposed cells rose from ~4 to >100 U/mg cellular protein, approaching a plateau. The amount of catalase responsible for the increased activity by a 2 h exposure corresponded to 2.5% of the cellular protein. In contrast, exposure to the control catalase (50 μg/ml) for 2 h only doubled the basal activity (Fig. 2A, inset). Immunoblotting of the same lysate with anti-Tat PTD antibody revealed similar kinetics of apparent transduction. By 60 min of exposure, the level of Tat-catalase protein had also approached a plateau (Fig. 2B). From the internal Tat-catalase standard on the same blot, it was estimated that the amount of Tat-catalase in the exposed cells accounted for between 4% and 8% of the total cellular proteins. Similar kinetics were observed for the transduction of HA Tat-catalase, although the increase in cellular enzyme activity was almost 3-fold higher than that with LA Tat-catalase (408 U/mg at 2 h), consistent with its 3-times higher enzyme activity.

In order to investigate the fate of the apparently transduced Tat-catalase, LA Tat-catalase-exposed cells (25 μg/ml, 2 h) were washed with PBS and incubated with fresh medium in the presence or absence of the proteasome inhibitor MG-132 (20 μM). The cellular catalase activity and anti-Tat antibody-detectable molecules declined with time following the end of exposure to the conjugate but both were maintained at a significantly higher level than baseline after the 4 h chase period (Figs. 2C and D). The presence of MG-132 during the chase period partially prevented the decay in cell-associated enzyme activity (Fig. 2C) as well as in anti-Tat antibody-detectable molecules (Fig. 2D), suggesting the involvement of a proteasome-dependent degradation process, although incubation with MG-132 did not result in accumulation of any appreciable amount of anti-Tat antibody-detectable degradation intermediates in either the lysates (Fig. 2D) or media (data not shown). Catalase activity, however, was detected in the medium 1 h after medium exchange and remained between 10% and 15% of the acquired activity over the 4 h chase period (Fig. 2C). Notably, the presence of MG-132 did not affect the reappearance of Tat-catalase in the medium (Fig. 2C). Thus, in L2 cells, after a 2 h incubation with LA Tat-catalase, 60% of the cell-associated enzyme conjugate was lost during the 4 h chase period, of which 10% appeared in the medium, 20% was degraded by an MG-132-inhibitable mechanism, likely proteasomal degradation, and 30% was lost through another mechanism, possibly lysosomal degradation and/or degradation by other proteases such as calpain (Fig. 2C). A similar metabolic fate was also observed with the same concentration of HA Tat-catalase, despite the difference in enzymatic activity of the conjugate in this cell line (data not shown) and in the A549-S cell line [21] (data not shown). To further evaluate how much Tat-catalase actually transduced into the cells during the 2 h exposure, HA Tat-catalase-exposed cells (25 μg/ml) were treated with trypsin. Trypsin treatment drastically reduced the enhanced cellular catalase activity to 18% of that of the trypsin-untreated cells (382–70 U/mg). Taken together, these data demonstrate that the great majority

of the apparently "transduced" Tat-catalase was actually tightly attached to the outer surface of the plasma membrane. This is in agreement with recent results showing that the PTD peptides bind avidly to the cell surface, due to their highly cationic nature, and remain attached even after extensive washing [14]. Our data show that some Tat-catalase was recovered in the medium during the chase experiment, which might suggest some reversibility of the membrane binding. Nonetheless, partial protection of Tat-catalase by the purported proteasome inhibitor suggests that a small portion of the conjugate must have entered the cells and potentially reached the cytosol. The next and most important question was to determine whether the cell-associated Tat-catalase could interfere with signaling pathways.

Effect of Tat-catalase on the activation of map kinase pathways

Growth factors, such as PDGF and EGF, as well as cell stressors, such as UV radiation, have been shown in some cells to cause H_2O_2 production, which in turn activate the mitogen-activated protein (MAP) kinases; i.e., the extracellular-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK. The activated MAP kinases then phosphorylate various substrates, among which are transcription factors (reviewed in (19)). Therefore, the activation of the ERK pathway by serum and the activation of the JNK pathway by anisomycin, a well-documented potent activator of JNK and p38 MAPK, were used to assess the Tat-catalase bioactivity.

In the absence of MG-132 (Fig. 3, upper four blots), although some inter- and intra-assay variability was observed, control catalase and HA Tat-catalase overall had no effect on the phosphorylation of ERK1/2 by serum (p-ERK, second blot from top). However, Tat-catalase caused partial inhibition, maximal at 10 μg/ml, of serum-induced phosphorylation of Elk (spot **a** in third blot), a transcription factor responsible for the transactivation of immediate early genes including c-fos and a downstream target for all three MAP kinase families [22]. In contrast, control catalase even at 50 μg/ml had only a marginal effect on Elk phosphorylation (spot **a** in third blot). Inhibition of serum-induced Elk phosphorylation by Tat-catalase without affecting ERK1/2 phosphorylation suggests that another kinase is responsible for Elk phosphorylation in L2 cells. Elk can be phosphorylated by p38 MAPK and JNK under stress stimulation [22,23] and by a ERK5-related kinase p97 [24]. Serum did not induce JNK activation in L2 cells, as its downstream substrate, c-Jun (see references therein [25]), was not phosphorylated. Thus, incubation of Tat-catalase with L2 cells demonstrated that the signaling pathway leading to serum-induced Elk phosphorylation was ERK- and JNK-independent but involved intracellular generation of H_2O_2.

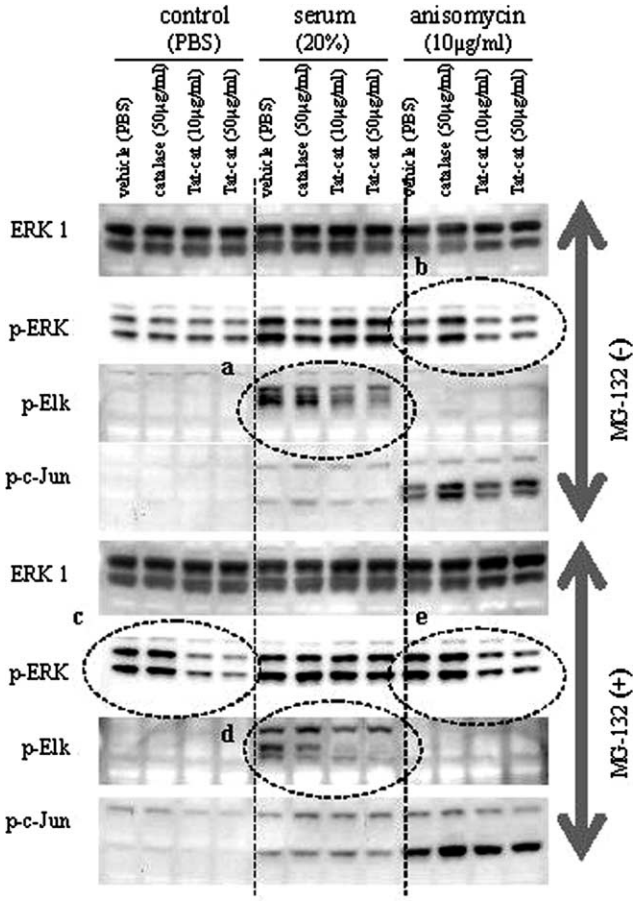

Fig. 3. Inhibitory profile of Tat-catalase on MAP kinase pathway activation. Serum-starved L2 cells were preincubated with either catalase (50 μg/ml) or HA Tat-catalase (10 or 50 μg/ml) for 2.5 h and then stimulated for 20 min with serum or anisomycin (catalase and Tat-catalase were present throughout stimulation). Cells were washed with PBS, lysed with 1× SDS–PAGE sample buffer, and analyzed by immunoblotting with the respective antibody. The blots shown here are representative of a total of five independent experiments conducted with HA and LA Tat-catalase. Note that Tat-catalase can inhibit serum-induced phosphorylation of Elk (**a** and **d**), anisomycin- and MG-132-induced phosphorylation of ERK (**b**, **c**, and **e**).

Tat-catalase had no effect on anisomycin-induced JNK activation, as assessed by phosphorylation of c-Jun, which was consistently detected as a doublet (fourth blot). Of note, despite JNK activation, anisomycin also failed to induce phosphorylation of Elk in L2 cells (third and seventh blots). Anisomycin has been shown to activate ERK in some cells, as it is the case here in L2 cells (second blot). Tat-catalase, but not catalase, inhibited anisomycin-induced phosphorylation of ERK (spot **b** in second blot). Thus, in these cells, anisomycin induced ERK activation through a pathway that appears to involve H_2O_2 production while that for JNK activation did not.

Since the proteasome inhibitor MG-132 partially prevented the degradation of the cell-associated Tat-catalase (Fig. 2), the effectiveness of Tat-catalase was also evaluated in the presence of 20 μM MG-132 (Fig. 3, lower

four blots). Interestingly, MG-132 alone induced the phosphorylation of ERK (compare controls of second and sixth blots). Inhibitors of the proteasome have been suggested to cause production of ROS [26] as well as activation of the ERK and JNK MAPK pathways [27]. In L2 cells, however, JNK was not activated by MG-132 and ERK, while activated, did not induce Elk phosphorylation. HA Tat-catalase at 10 μg/ml maximally inhibited MG-132-induced phosphorylation of ERK to basal level, in agreement with a role for ROS in ERK activation by MG-132 while control catalase had no effect (spot **c** in sixth blot). Activation of ERK1/2 by serum in the presence of MG-132 was not affected by catalase or Tat-catalase but the inhibitory action of Tat-catalase against serum-induced phosphorylation of Elk was potentiated as 10 μg/ml Tat-catalase now completely inhibited the serum-induced phosphorylation of Elk (spot **d** in seventh blot), suggesting that the Tat-catalase localized in a proteasome-accessible compartment was in part responsible for this inhibition. Tat-catalase also inhibited anisomycin-induced phosphorylation of ERK in the presence of MG-132 (spot **e** in sixth blot), although the inhibition was not potentiated. As previously, neither catalase nor Tat-catalase had any effect on c-Jun phosphorylation in the presence of MG-132.

Experiments using LA Tat-catalase demonstrated an almost identical inhibitory profile but with a slightly lesser potency than HA Tat-catalase (data not shown). As minute amounts of mica-Tat peptide inevitably contaminated the Tat-catalase preparation (see Materials and methods), we also measured the effect of mica-Tat peptide on MAPK phosphorylation. A concentration of 0.1 μM mica-Tat peptide, corresponding to approximately 50% equimolar and 10% equimolar Tat-catalase at 10 and 50 μg/ml, respectively, had neither an inhibitory nor a stimulatory effect at all on MAPK phosphorylation events (data not shown). Thus, the effectiveness of Tat-catalase is mediated by the Tat-catalase conjugate and not by a contaminating mica-Tat peptide.

Conclusions

The present study show that Tat-catalase was mostly tightly bound to the surface of L2 cells, thus confirming recent reports [12–14]. Nevertheless, the data also shows that Tat-catalase could inhibit intracellular signaling, contrary to native catalase that was ineffective on the same pathways. We cannot definitely conclude whether the Tat-catalase operative on signaling was that bound to the plasma membrane or the small fraction that had entered the cells, although the lack of effect of native catalase suggests that close proximity to the source of production of H_2O_2 is required for catalase efficiency and favors a role for intracellular catalase. Moreover, the increased effectiveness of Tat-catalase on Elk phosphorylation (Fig. 3, spot **d**) in the presence of MG-132 suggests that the fraction of Tat-catalase located in a compartment accessible to MG-132, probably the cytosol, is in part responsible for this effect. Yet, as most of the cell-associated catalase was not degraded by the proteasome, Tat-catalase must also be localized to other compartments. Lundberg et al. [12] while demonstrating that influx of VP22 protein occurs during cell fixation, postulated that the PTD-proteins adhering to the cell membrane could end up into endosomes and lysosomes. Others have reported that fluorescence microscopic examination of living cells incubated with Tat-PTD conjugates revealed a punctate distribution of these peptides, characteristic of endocytosis [14]. Thus, it is conceivable that some of the Tat-catalase in our study was located to the inner surface of endosomes/lysosomes where it could also be effective in scavenging membrane permeable H_2O_2, thereby modulating signaling. While further in-depth research will be needed to identify the H_2O_2-dependent steps in the MAPK pathways and to clarify the inhibitory mechanism of Tat-catalase on these pathways, Tat-catalase can be added to the expanding list of Tat-modified molecules that exert a biological effect and is clearly a novel tool that can be used to dissect H_2O_2-dependent signal transduction pathways.

Acknowledgments

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