Serine protease inhibitors suppress cytochrome *c*-mediated caspase-9 activation and apoptosis during hypoxia–reoxygenation

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We have shown that reoxygenation of hypoxic rat kidney proximal tubule cells leads to apoptosis. This is mediated by translocation of Bax from the cytosol to mitochondria, accompanied by release of mitochondrial cytochrome *c* (cyt.*c*). The present study has examined the proteolytic mechanisms responsible for apoptosis during hypoxia–reoxygenation. Caspases were activated during hypoxia, as shown by cleavage of fluorogenic peptide substrates. By 5 h caspase-3-like activity to cleave carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin was increased approx. 30-fold. This was accompanied by specific processing of pro-caspase-3, -8 and -9 into active forms. Caspase activation during hypoxia was blocked by carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone and overexpression of Bcl-2. Of particular interest, caspase activation was also suppressed by the chymotryptic inhibitors *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and Ala-Pro-Phe chloromethyl ketone (APF), and the general serine protease inhibitor 4-(2-aminoethyl)benzenesulphonyl fluoride. Inhibition of caspase activation by these compounds resulted in arrest of apoptosis. On the other hand, the serine protease inhibitors did not prevent release of mitochondrial cyt.*c* during hypoxia, suggesting that these compounds blocked a critical step in post-mitochondrial caspase activation. Further studies using an *in vitro* reconstitution model showed that cyt.*c*/dATP stimulated caspase-9 processing and downstream caspase activation were significantly suppressed in the presence of TPCK and APF. Based on these results, we speculate that serine proteases may be involved in post-mitochondrial apoptotic events that lead to activation of the initiator, caspase-9.

Key words: apoptosis, apoptosome, ATP depletion, reoxygenation, TPCK.

INTRODUCTION

Proteolytic mechanisms are major effectors of the form of cell death known as apoptosis. The caspase family of cysteine proteases, which cleave substrates after aspartic acid residues, mediate the key proteolytic events responsible for the initiation, as well as execution, of apoptosis [1–4]. Although it is well established that caspases play central roles in apoptotic proteolysis, there exists a body of evidence suggesting that serine proteases may also be involved [5,6]. Inhibitors of serine proteases were found to suppress apoptosis [7–12], but the critical steps at which they act remain to be determined.

The current study has examined proteolytic events responsible for apoptosis caused by hypoxia–reoxygenation. We have previously reported that hypoxia leads to translocation of the pro-apoptotic protein Bax from the cytosol to mitochondria, release of cytochrome *c* (cyt.*c*) from mitochondria into the cytosol and caspase activation, followed by apoptosis during reoxygenation [13,14]. Here we show that both of the initiator caspases, caspase-8 and -9, as well as the executioner caspase-3, are processed into enzymically active forms during hypoxia, following the release of cyt.*c*. Much to our surprise, the chymotryptic inhibitors *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and Ala-Pro-Phe chloromethyl ketone (APF) and the general active-site-directed serine protease inhibitor 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF) not only suppressed apoptosis, but also abolished the processing and activation of all caspases entirely. Since the serine protease inhibitors did not prevent the release of mitochondrial cyt.*c*, our results suggest that these reagents blocked a critical step of cyt.*c*-mediated caspase activation. Further experiments using an *in vitro* reconstitution model demonstrated that activation of caspase-9 and downstream events stimulated by cyt.*c*/dATP in cytosolic extracts were significantly suppressed by TPCK and APF. These studies suggest that serine proteases may have a role in early post-mitochondrial steps required to initiate the apoptotic cascade.

MATERIALS AND METHODS

Materials

Rat kidney proximal tubule cells (SKPT-0193, clone 2) were provided by Dr. U. Hopfer (Case Western Reserve University, Cleveland, OH, U.S.A.). Reagents were from the following sources: APF, inhibitors of caspases and fluorogenic peptide substrates used for caspase activity assays were from Enzyme Systems Products (Dublin, CA, U.S.A.); MG132, lactacystin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from Calbiochem–Novabiochem Co. (San Diego, CA, U.S.A.);

Abbreviations used: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; AFC, 7-amino-4-trifluoromethyl coumarin; APF, Ala-Pro-Phe chloromethyl ketone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cyt.*c*, cytochrome *c*; DCI, 3,4-dichloroisocoumarin; DEVD.AFC, carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; DTT, dithiothreitol; FA, carbobenzoxy-Phe-Ala-fluoromethyl ketone; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; VAD, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone.

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polyclonal antibodies to caspase-1 (M-19, recognizing pro-caspase-1 and its p20 subunit), caspase-2 (N-19, recognizing pro-caspase-2 and its p20 subunit), caspase-3 (K-19, recognizing pro-caspase-3 and its p10 subunit), caspase-4 (N-15, recognizing pro-caspase-4 and its p20 subunit), caspase-6 (A-16, recognizing pro-caspase-6 and its p10 subunit), caspase-7 (C-18, recognizing pro-caspase-7 and its p10 subunit), caspase-8 (T-16, recognizing pro-caspase-8 and its p20 subunit), gelsolin (N-18) and lamin B (M-20) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.); the polyclonal antibody to caspase-9 recognizing pro-caspase-9 and its large subunit was from Idun Pharmaceuticals Inc. (La Jolla, CA, U.S.A.); the monoclonal antibody to cyt.*c* (Clone 7H8.2C12) was from PharMingen (San Diego, CA, U.S.A.) and the monoclonal antibody to native cyt.*c* (Clone 2G8.B6) was from Dr. R. Jemmerson (University of Minnesota Medical School, Minneapolis, MN, U.S.A.). All other reagents were obtained from Sigma (St. Louis, MO, U.S.A.).

Experimental model

An *in vitro* model of hypoxia–reoxygenation has been described previously [13], and was adopted in this study. Briefly, cells were washed with PBS, transferred to an anaerobic chamber with N_2/CO_2 (19:1), and incubated in Krebs-Ringer bicarbonate buffer. This buffer was pregassed with N_2/CO_2 (19:1). EC Oxyrase, a biocatalytic oxygen reducing agent, was added to the medium (1.2 units/ml), to consume residual O_2 and maximize the degree of hypoxia. Also included in the hypoxic medium was 5 mM glycine, which simulated glycine contents of ischaemic tissues *in vivo*, and thus prevented early necrotic injury during hypoxic incubation [15]. After hypoxia, cells were transferred back to full culture medium (serum supplemented Ham's medium F12/ Dulbecco's modified Eagle's medium) in air/CO_2 (19:1) for reoxygenation.

Measurement of caspase activity

The enzymic activity of caspases was measured by monitoring the cleavage of exogenous fluorogenic peptide substrates, yielding 7-amino-4-trifluoromethyl coumarin (AFC) as the fluorescent signal [16,17]. Following incubation, dishes with cells were placed on ice. Floating cells and debris in the medium were collected by centrifugation and combined with the cells remaining in the dishes. Cells were subsequently lysed with lysis buffer [1% (v/v) Triton X-100/115 mM NaCl/1 mM KH_2PO_4/4 mM KCl/ 1 mM dithiothreitol (DTT)/25 mM Hepes, pH 7.4] containing 1 mM benzamidine, 1 mM PMSF, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin. The resulting lysates were centrifuged at 12000 ***g*** for 5 min at 4 °C to obtain supernatants. The supernatants (50 μg of protein in 50 μl) were added to 200 μl of enzymic reaction buffer (0.1% CHAPS/10% sucrose/1 mM EDTA/10 mM DTT/100 mM Hepes, pH 7.4) containing 50 μM peptide substrate. The reaction was allowed to proceed for 60 min at 37 °C. Fluorescence was subsequently monitored, by exciting the samples at 360 nm and measuring the emission at 530 nm, on a SpectroFluor plate reader (Tecan US Inc., Research Triangle Park, NC, U.S.A.). Background fluorescence was determined with 50 μl of lysis buffer and 200 μl of reaction buffer containing 50 μM peptide substrate, and subtracted from the reaction values. For each measurement, a standard curve was constructed with free AFC. Based on the standard curve, the fluorescence reading from the enzymic reaction was translated into the molar amount of liberated AFC.

Immunoblot analysis

At the end of incubation, cells were dissolved in sample buffer containing 2% (w/v) SDS, 100 mM DTT and 62.5 mM Tris/HCl (pH 6.8). Proteins in whole cell lysates were subjected to reducing SDS/PAGE (4–12% polyacrylamide). The resolved proteins were then electroblotted onto PVDF membranes. After 1 h of blocking in 2% BSA, the membranes were incubated overnight with primary antibodies at 4 °C. The following morning, the membranes were thoroughly washed and incubated with horseradish-peroxidase-conjugated secondary antibodies. Antigens on the membranes were finally revealed by exposure to chemiluminescent substrates (Pierce, Rockford, IL, U.S.A.).

Immunohistochemical localization of cyt.*c*

Cells were fixed with a modified Zamboni's fixative (4% paraformaldehyde/0.19% picric acid in PBS, pH 7.4) for 1 h at room temperature. Fixed cells were sequentially washed with PBS and PBS containing 0.1 M glycine. After 5 min of permeabilization with 0.1% SDS, cells were washed and subjected to 1 h of blocking with 5% (v/v) normal goat serum. The cells were washed again and incubated with a monoclonal antibody against native cyt.*c* (1:400, diluted in PBS) for 1 h at room temperature. After exposure to the primary antibody, cells were incubated again with 5% normal goat serum. Antigenic sites were revealed by secondary staining with CY3-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, U.S.A.) and examined by fluorescence microscopy.

In vitro reconstitution of cyt.*c*/dATP stimulated caspase activation

Cytosolic extracts were prepared by selective permeabilization of the plasma membrane with digitonin [13]. Briefly, cells were washed with PBS and exposed to 0.05% digitonin in isotonic buffer (250 mM sucrose/10 mM Hepes/10 mM KCl/1.5 mM $MgCl_2$/1 mM EDTA/1 mM EGTA, pH 7.1) for 1 min at room temperature. Soluble fractions were collected and centrifuged at 12000 ***g*** for 10 min. Proteins in the resultant supernatants were concentrated to 4–5 mg/ml with 3K cutoff microconcentrators (Pall Filtron Co., Northborough, MA, U.S.A.) and kept frozen at −70 °C. Typical reconstitutions were set up in volumes of 10 or 20 μl. For 10 μl reconstitutions, 1 μl of 0.5 mg/ml rat heart cyt.*c* and 1 μl of 10 mM dATP were added to 7.5 μl of cytosolic extracts, containing 25 μg of protein, and incubated for 1–2 h at 30 or 37 °C. After incubation, the reconstitution mixtures were subjected to SDS/PAGE for immunoblotting, or transferred to 200 μl of enzymic reaction buffer containing 50 μM DEVD.AFC (carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin) to determine caspase activity. To test the effects of serine protease inhibitors, cytosolic extracts were preincubated with the inhibitors for 10 min at 30 °C prior to addition of cyt.*c*/dATP.

RESULTS

Caspase activation during hypoxia

Reoxygenation of hypoxic rat kidney proximal tubule cells led to cell death by apoptosis. This was shown by the development of apoptotic morphology, breakdown of nuclear lamins and DNA degradation [13]. To investigate proteolysis in these cells, we initially examined caspase activity using fluorogenic peptide substrates. The peptide substrates are conjugated with AFC, and have aspartic acid residues at P1 positions, a requirement for

Table 1 Activation of caspases during hypoxia

Cells (Con, control) were subjected to 1, 3 or 5 h of hypoxic incubation in the absence of metabolic substrates (1H, 3H and 5H), or 5 h of hypoxia followed by 2 h of reoxygenation (2R). VAD, 100 μM VAD was provided during 5 h of hypoxia; FA, 100 μM FA was provided during 5 h of hypoxia; Bcl-2, 5 h of hypoxia was carried out with cells stably transfected with a Bcl-2 vector. Following incubation, cells were extracted with a buffer containing 1% Triton X-100. Cell lysates (50 μg of protein) were added to enzymic reactions containing one of the fluorogenic peptide substrates (see text for the abbreviations). After 60 min of incubation at 37 °C, fluorescence was monitored by exciting the samples at 360 nm and measuring the emission at 530 nm, in order to determine the production of free AFC (nmol). Values are means ± S.E.M. ($n = 4$).

Substrate (caspase)	YVAD (1,4)	DEVD (3,6,7,8,10)	VDVAD (2)	VEID (6)	IETD (8)	LEHD (9)
Con	0.02 ± 0.01	0.13 ± 0.04	0.08 ± 0.02	0.09 ± 0.00	0.03 ± 0.00	0.05 ± 0.00
1H	0.04 ± 0.01	0.35 ± 0.02	0.14 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.14 ± 0.00
3H	0.03 ± 0.02	1.67 ± 0.07	0.52 ± 0.04	0.36 ± 0.03	0.23 ± 0.01	0.37 ± 0.03
5H	0.04 ± 0.02	3.74 ± 0.09	0.99 ± 0.09	0.85 ± 0.03	0.39 ± 0.02	0.54 ± 0.05
2R	0.03 ± 0.01	3.69 ± 0.13	0.74 ± 0.07	0.61 ± 0.08	0.29 ± 0.02	0.39 ± 0.03
VAD	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.08 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
FA	0.03 ± 0.02	3.46 ± 0.14	0.95 ± 0.08	0.87 ± 0.09	0.42 ± 0.04	0.49 ± 0.06
Bcl-2	0.04 ± 0.02	0.54 ± 0.05	0.19 ± 0.06	0.18 ± 0.01	0.08 ± 0.00	0.09 ± 0.01

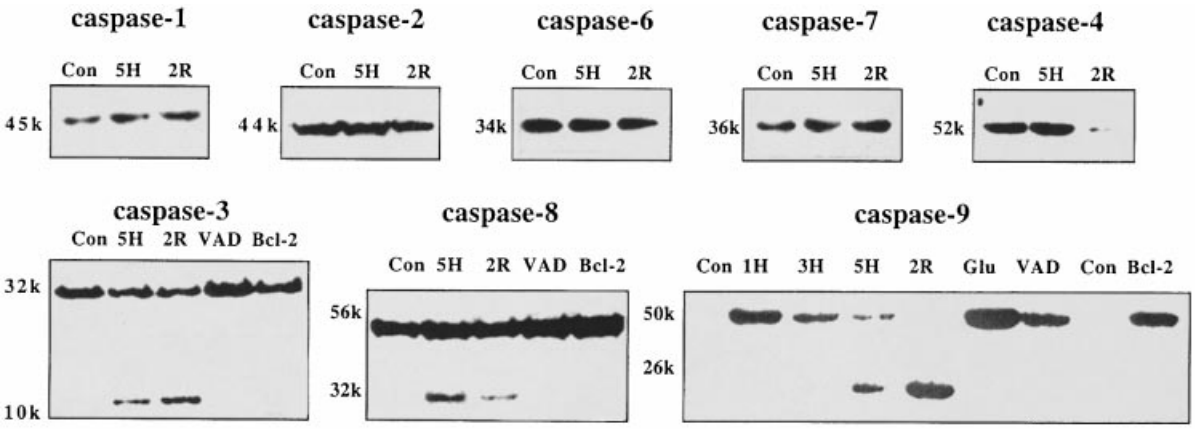

Figure 1 Processing of caspases during hypoxia–reoxygenation

Cells (Con, control) were subjected to 1, 3 or 5 h of hypoxic incubation in the absence of metabolic substrates (1H, 3H and 5H), or 5 h of hypoxia followed by 2 h of reoxygenation (2R). VAD, 100 μM VAD was provided during 5 h of hypoxia; Bcl-2, 5 h of hypoxia was carried out using cells overexpressing Bcl-2; Glu, 5.5 mM glucose was provided during 5 h of hypoxia. Following incubation, cells were lysed in sample buffer containing 2% SDS. Proteins in whole-cell lysates (100 μg/lane) were resolved by SDS/PAGE, electroblotted onto PVDF membranes, and probed with antibodies specific for caspase-1, -2, -3, -4, -6, -7, -8 or -9.

caspase proteolysis. Cleavage of these substrates after the aspartic acid residue results in release of AFC, a fluorescent product. We measured enzymic activities using six AFC-conjugated peptide substrates, with sequences preferred by specific caspases. The results are summarized in Table 1. The enzymic activities for YVAD.AFC, a preferred substrate for caspase-1 and caspase-4, were barely detectable in control cells, and remained minimal throughout hypoxia and reoxygenation, suggesting that there was no activation of these two caspases. In contrast, hypoxia led to progressive increases in enzymic activity cleaving the caspase substrates DEVD.AFC, VDVAD.AFC, VEID.AFC, IETD.AFC and LEHD.AFC, implying possible activation of caspase-2, -3, -6, -7, -8, -9 and -10 [16–18]. Maximal activity was detected with DEVD.AFC. After 5 h of hypoxia, DEVDase activity increased by approx. 30-fold (compared with the control). Reoxygenation did not result in further elevation. Activation of caspases during hypoxia was blocked by VAD (carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone), an active-site-directed inhibitor of caspases [19]. Carbobenzoxy-Phe-Ala-fluoromethyl ketone (FA), an inhibitor of non-caspase cysteine proteases, was without effect. Moreover, caspase activation was abrogated by overexpression of Bcl-2, an anti-apoptotic protein, consistent with the cytoprotective action of this protein during hypoxia–reoxygenation [13].

Processing of caspase-3, -8 and -9 into active forms during hypoxia

Our studies with fluorogenic peptide substrates demonstrated the activation of caspases by hypoxia. Based on the peptide sequences, the activation could occur in caspase-2, -3, -6, -7, -8, -9 and -10 but not in caspase-1 or -4. Since caspases may be promiscuous with respect to substrate specificity, it was difficult to determine which specific enzymes were really activated. Activation of caspases requires proteolytic processing [1–4]. Thus, to identify the activated caspases, we subsequently examined changes of the enzymes during hypoxia–reoxygenation by immunoblotting with specific antibodies. The results are shown in Figure 1. No alterations were detected in caspase-1, -2, -6 and -7 after hypoxia or reoxygenation (Figure 1). Pro-

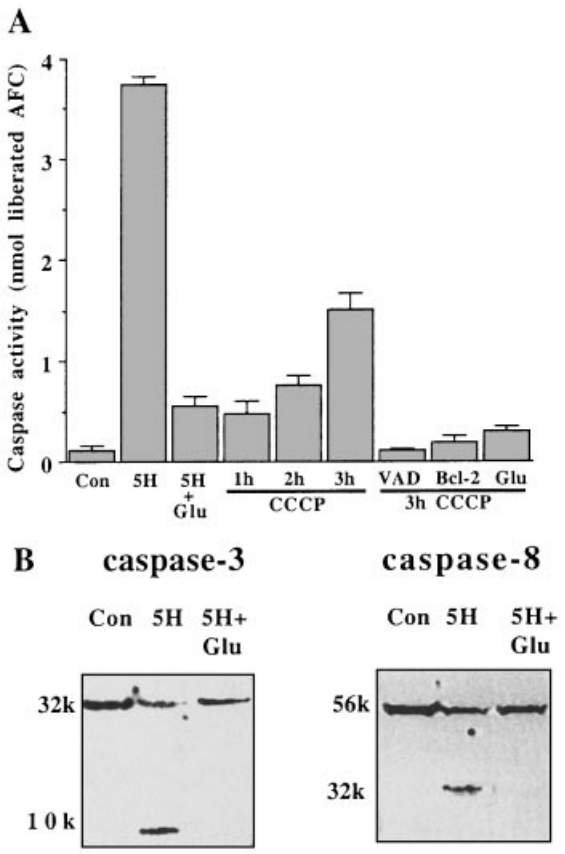

Figure 2 Role for ATP depletion in caspase activation during hypoxia

(**A**) Fluorometric assay of caspase activity. Cells (Con, control) were subjected to 5 h of hypoxia without (5H) or with 5.5 mM glucose (5H + glu), or 1, 2 or 3 h of incubation with 15 μM CCCP. VAD, 100 μM VAD was provided during 3 h of CCCP incubation; Bcl-2, 3 h of CCCP incubation was carried out with cells overexpressing Bcl-2; Glu, 5.5 mM glucose was provided during 3 h of CCCP incubation. Following incubation, cell lysates were extracted and caspase activity was measured by enzymic reactions with DEVD.AFC, as described in the Materials and methods section. Results are means ± S.E.M. ($n = 4$). (**B**) Immunoblot analysis of caspase-3 and -8. Cells (Con, control) were subjected to 5 h of hypoxia without (5H) or with 5.5 mM glucose (Glu), and lysed with SDS sample buffer. Proteins in whole-cell lysates were analysed by immunoblotting using antibodies specific for caspase-3 or -8.

caspase-4 remained intact during hypoxia, and was degraded in reoxygenated cells (Figure 1). However, no specific fragments were detected (results not shown). Typical proteolytic processing was revealed for caspase-3, -8 and -9, resulting in the generation of fragments with apparent sizes of 10, 32 and 26 kDa, respectively (Figure 1). Formation of these fragments was evident only after 5 h of hypoxia, although increases in enzymic activities were detected at earlier time points (Table 1). We attributed these results to the lower sensitivity of the immunoblotting technique in detecting activated caspases relative to the enzymic activity assay. Fewer cells might be expected to display caspase activation after 1 or 3 h of hypoxia, based on the numbers of cells that had leaked cyt.*c* from their mitochondria at these intervals [13]. Processing of the caspases was completely attenuated by VAD and overexpression of Bcl-2 (Figure 1). Notably, caspase-9 was not detectable in control cells, and was induced during hypoxia. The up-regulated enzyme was subsequently processed into an active form after hypoxic incubation (Figure 1). Since caspase-9 was detected only after hypoxia, and its processed form was smaller than the p35/p37 seen in human cells, we verified the identity of rat caspase-9 with antibodies recognizing different epitopes prepared by Santa Cruz Biotechnology Inc. and by our laboratory (results not shown).

Table 2 Effect of protease inhibitors on caspase activation during hypoxia

Cells were subjected to 5 h of hypoxic incubation in the absence of metabolic substrates without (–) or with various protease inhibitors. Following incubation, cells were extracted with a buffer containing 1% Triton X-100. Cell lysates (50 μg of protein) were added to an enzymic reaction containing the fluorogenic substrate, DEVD.AFC. After 60 min of incubation at 37 °C, fluorescence was monitored by exciting the samples at 360 nm and measuring the emission at 530 nm, in order to determine the production of free AFC. Values are means ± S.E.M. ($n = 4$).

Inhibitor	Concentration (μM)	Caspase activity (nmol liberated AFC)
–	–	3.35 ± 0.14
VAD*	100	0.10 ± 0.04
E64d	100	2.11 ± 0.50
Leupeptin	100	3.17 ± 0.12
Pepstatin	100	3.14 ± 0.30
PMSF	1000	3.22 ± 0.17
AEBSF*	1000	0.21 ± 0.02
DCI	10	3.65 ± 0.15
TPCK*	40	0.21 ± 0.05
APF*	20	0.04 ± 0.02
TLCK	40	3.12 ± 0.26
Phenanthroline	2500	3.61 ± 0.50
EDTA	5000	3.19 ± 0.66
MG132	100	2.70 ± 0.27
Lactacystin	10	3.26 ± 0.12

* chemicals showing remarkable inhibitory effects.

Caspase activation during hypoxia requires ATP depletion

During hypoxic incubation without glucose, cells are exposed to two types of immediate stress, i.e. lack of oxygen and deprivation of cellular ATP. Many pathologic processes are initiated during hypoxia by ATP depletion, rather than by oxygen deficiency directly [20]. In our experimental model of hypoxia, progressive decreases of cellular ATP were demonstrated. After 5 h of hypoxic incubation in the absence of metabolic substrates, cell ATP was severely depleted, to near zero levels [13]. To investigate the role of ATP depletion in the observed caspase activation during hypoxia, we initially tested the effects of glucose, a glycolytic substrate. When 5.5 mM glucose was provided during 5 h of hypoxia, cell ATP was maintained at substantial levels, approx. 70% of control (results not shown). In these cells, caspase activation was suppressed (Figure 2A, 5H + Glu). Concomitantly, processing of caspase-3, -8 and -9 was blocked [Figures 2B and 1 (caspase-9)]. To further examine the involvement of ATP depletion, we incubated cells under a normal oxygen atmosphere in glucose free medium containing CCCP, a mitochondrial uncoupler. ATP levels in CCCP-treated cells declined rapidly (results not shown). This was accompanied by progressive increases of caspase activity, as shown in Figure 2A. As in the case of hypoxia, caspase activation induced by CCCP was completely blocked by VAD and Bcl-2 overexpression (Figure 2A). Moreover, provision of glucose, which generated glycolytic ATP during CCCP treatment, also attenuated caspase activation (Figure 2A). Together, the results point to a critical role for ATP depletion in caspase activation triggered by hypoxia.

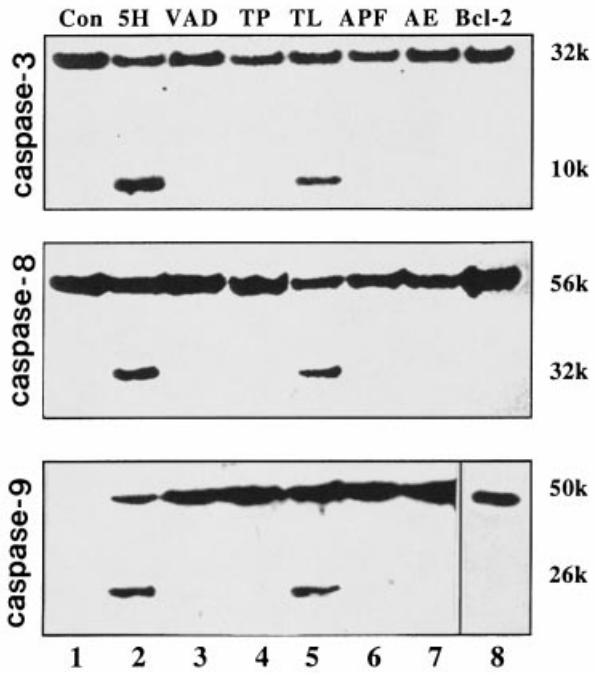

Figure 3 Inhibition of caspase processing in hypoxic cells by TPCK, APF and AEBSF

Cells (Con, control) were subjected to 5 h of hypoxic incubation in the absence of metabolic substrates without (5H), or with various protease inhibitors. VAD, 100 μM VAD; TP, 40 μM TPCK; TL, 40 μM TLCK; APF, 20 μM APF; AE, 1 mM AEBSF; Bcl-2, 5 h of hypoxia was carried out with cells overexpressing Bcl-2. Following incubation, cells were dissolved in SDS sample buffer. Proteins in whole-cell lysates were analysed by immunoblotting using antibodies specific for caspase-3, -8 or -9. *Lane 8 of the caspase-9 immunoblot was from a separate experiment.

Serine protease inhibitors suppress caspase activation during hypoxia

Despite the central role played by caspases, proteases of other classes have been implicated in apoptosis [5,6]. This is suggested by pharmacological studies using inhibitors [7–12], and supported by evidence showing the ability of non-caspase proteases to process and activate caspases [21]. To investigate the possible participation of proteolytic enzymes other than caspases in apoptotic mechanisms triggered by hypoxia, we examined the effects of inhibitors targeting various classes of proteases. The agents tested were as follows: caspase inhibitor VAD; the general cysteine protease inhibitors E64d and leupeptin; the general aspartic protease inhibitor pepstatin; the general active-site serine protease inhibitors PMSF, 3, 4-dichloroisocoumarin (DCI), and AEBSF; chymotrypsin-like serine protease inhibitors TPCK and APF; trypsin-like serine protease inhibitor *N*-tosyl-L-lysine chloromethyl ketone (TLCK); metalloprotease inhibitors 1,10-phenanthroline and EDTA and proteasome inhibitors MG132 and lactacystin [22,23]. The results are summarized in Table 2. The inhibitors were used at concentrations known to be effective against prototypic proteases, and were not noticeably cytotoxic during hypoxia. VAD at 100 μM completely blocked caspase activation during hypoxia, consistent with previous observations. Among the other inhibitors tested, TPCK, APF and AEBSF exhibited significant inhibition, E64d and MG132 showed marginal effects, while the others were without effect. Suppression of caspase activation by TPCK, APF and AEBSF was further supported by their effects on caspase processing. As shown in Figure 3, cleavage of caspase-3, -8 and -9 to yield 10, 32 and 26 kDa fragments, respectively, was evident after 5 h of hypoxia (lane 2), and this was totally abolished by TPCK, APF and AEBSF (lanes 4, 6 and 7, respectively). In contrast, other inhibitors, including TLCK, were without effect (Figure 3, lane 5).

Inhibition of apoptosis during reoxygenation by serine protease inhibitors

Typical apoptotic morphology develops in reoxygenated rather than hypoxic cells [13], despite the demonstrated caspase activation during hypoxia. This is probably due to the ATP requirement for completion of apoptotic programs. The inhibitory effects of TPCK, APF and AEBSF on caspase activation in hypoxic cells prompted us to follow the outcome with respect to the development of apoptosis in these cells after reoxygenation. As shown in Figure 4, reoxygenation of hypoxic cells led to cellular condensation, nuclear fragmentation and formation of apoptotic bodies (Figure 4, 5H/1R). TPCK inhibited development of these apoptotic features completely (Figure 4, 5H/1R + TPCK). Inhibitory effects were also demonstrated for APF and AEBSF, although these two reagents showed higher cytotoxicity. Although all three inhibitors prevented apoptosis during reoxygenation, their long-term cytotoxic effects resulted in eventual loss of viability by necrosis (results not shown). Unlike the chymotryptic inhibitors, TPCK and APF, the tryptic inhibitor TLCK did not mitigate the progress of apoptosis during reoxygenation (Figure 4, 5H/1R + TLCK), consistent with its lack of effect on caspase activation (Figure 3).

To provide support for these morphological observations, we analysed apoptotic breakdown of structural cell proteins. Gelsolin and lamin B have been shown to be fragmented in diverse apoptotic models [1–4], and were chosen in our study to represent alterations of cytosolic and nuclear proteins. The results are shown in Figure 5. Gelsolin and lamin B remained largely intact during 5 h of hypoxic incubation (lane 2). After 1 h of reoxygenation, these two structural proteins were cleaved, resulting in the formation of 46 and 48 kDa fragments, respectively (lane 3). As expected, fragmentation of these proteins was inhibited by Bcl-2, glucose (lanes 8, 9) and VAD (results not shown). Significantly, TPCK, APF and AEBSF abolished the breakdown of these proteins as well (lanes 4, 6 and 7, respectively), while TLCK was without effect (lane 5).

Serine protease inhibitors do not target caspases directly

Our results have demonstrated inhibitory effects of TPCK, APF and AEBSF on caspase activation during hypoxia, resulting in arrest of apoptosis in reoxygenated cells. An immediate question which arose was: are these inhibitors acting directly on caspases that have already been activated? To answer this, we tested the effects of these inhibitors on the enzymic activity of activated caspases. For this purpose, cytosol with fully activated caspases was extracted from hypoxic cells, preincubated with various inhibitors, and added to the enzymic reaction containing the fluorogenic substrate DEVD.AFC. The results are shown in Figure 6. TPCK and APF failed to inhibit caspase activity significantly at concentrations up to 200 μM, well above that required to block caspase activation in hypoxic cells (40 μM). In contrast, VAD abolished caspase activity at 1 μM. The results do not support direct inhibition of active caspases by serine protease

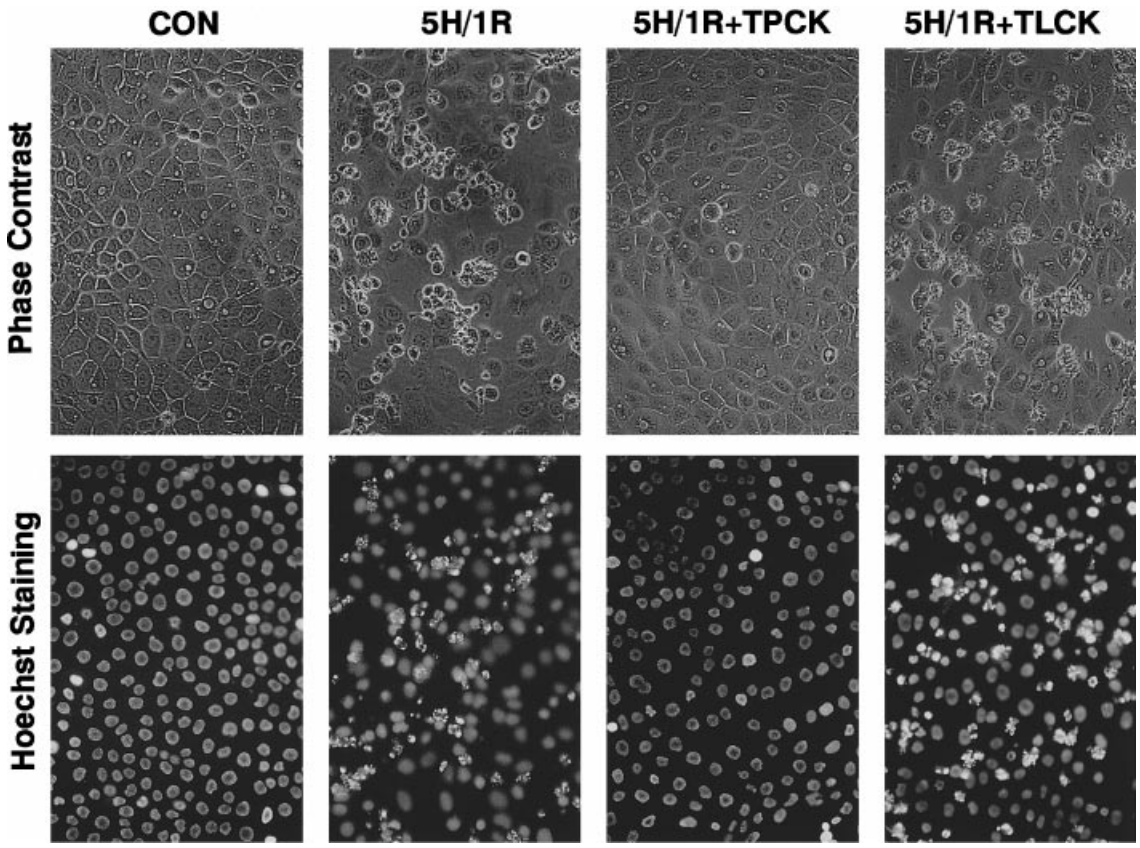

Figure 4 Suppression of apoptotic morphology in reoxygenated cells by TPCK

Cells (CON, control) were subjected to 5 h of hypoxia followed by 1 h of reoxygenation without (5H/1R), or with 40 μM TPCK (5H/1R + TPCK) or 40 μM TLCK (5H/1R + TPCK). Following incubation, cells were stained with 10 μg/ml Hoechst 33342 for 2 min at 37 °C. Cell morphology and nuclear staining of the same fields were recorded by phase contrast and fluorescence microscopy, respectively.

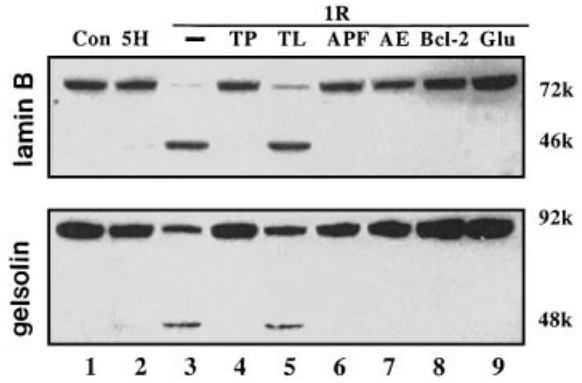

Figure 5 Suppression of proteolysis of gelsolin and lamin B during hypoxia–reoxygenation by TPCK, APF and AEBSF

Cells (Con, control) were subjected to 5 h of hypoxia alone (5H), or 5 h of hypoxia followed by 1 h of reoxygenation (1R). Protease inhibitors were provided throughout 5 h of hypoxia/1 h of reoxygenation: –, no inhibitors; TP, 40 μM TPCK; TL, 40 μM TLCK; APF, 20 μM APF; AE, 1 mM AEBSF; Bcl-2, cells overexpressing Bcl-2; Glu, 5.5 mM glucose. Following incubation, cells were dissolved in SDS sample buffer. Proteins in whole-cell lysates were analysed by immunoblotting using antibodies specific for lamin B and gelsolin.

inhibitors; instead, they suggest interference at upstream steps initiating caspase activation during hypoxia.

Serine protease inhibitors do not block cyt.*c* translocation during hypoxia

Two major pathways leading to caspase activation during apoptosis have been documented [24,25]. While one pathway is mediated by death receptors, the other is initiated by cytosolic cyt.*c* released from mitochondria. Our recent studies have demonstrated leakage of mitochondrial cyt.*c* in hypoxic cells. Upon reoxygenation, cells with leaked cyt.*c* underwent apoptosis [13]. Overexpression of Bcl-2 blocked cyt.*c* translocation and death of reoxygenated cells. The results point to a critical role for cyt.*c*-mediated apoptotic pathways in cell injury during hypoxia–reoxygenation. This raised the possibility that serine protease inhibitors might suppress caspase activation during hypoxia by blocking the release of mitochondrial cyt.*c*. To examine this issue, we fractionated cells into cytosolic and membrane-bound fractions by selective plasma membrane permeabilization with digitonin. The membrane-bound fraction contains mitochondria [13]. These two fractions were analysed for cyt.*c* by immunoblotting. The results are shown in Figure 7. In control cells, cyt.*c* was exclusively mitochondrial (lane 1). After 5 h of hypoxia, cyt.*c* in the mitochondrial fraction was decreased, and this was

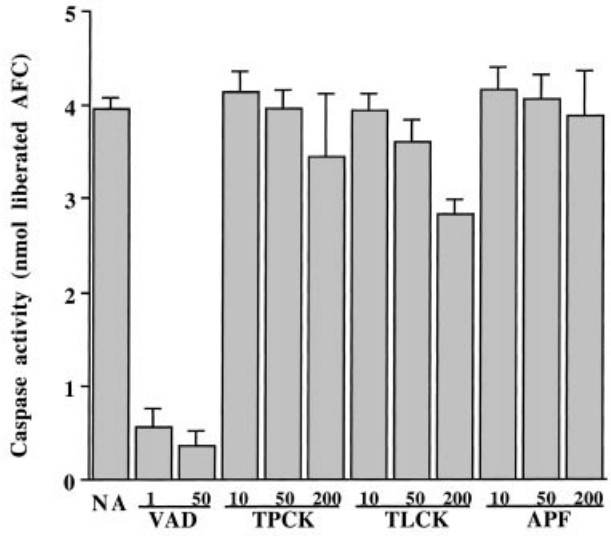

Figure 6 ***In vitro*** **effects of serine protease inhibitors on activated caspases**

Cells were subjected to 5 h of hypoxic incubation without metabolic substrates in order to activate caspases. The hypoxic cells were subsequently extracted with a buffer containing 1% Triton X-100. The resultant cell lysate (50 μg protein) containing activated caspases was pre-incubated at 37 °C for 5 min without (NA) or with protease inhibitors, and then added to enzymic reactions containing DEVD.AFC, a fluorogenic substrate for caspases. Caspase activity was measured as described in the Materials and methods section. Concentrations of inhibitors tested were: 1 and 50 μM for VAD; 10, 50 and 200 μM for TPCK, TLCK and APF. Results are means $\pm$ S.E.M. ($n = 4$).

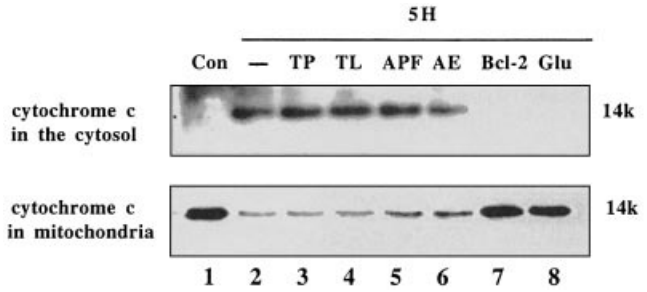

Figure 7 Immunoblot analysis of cyt.*c* translocation in hypoxic cells

Cells (Con, control) were subjected to 5 h of hypoxic incubation in the absence of metabolic substrates (5H). Agents provided during hypoxia were: —, no addition; TP, 40 μM TPCK; TL, 40 μM TLCK; APF, 20 μM APF; AE, 1 mM AEBSF; Glu, 5.5 mM glucose. Bcl-2, hypoxia was carried out with cells overexpressing Bcl-2. Following incubation, cells were sequentially extracted with digitonin and Triton X-100. Digitonin lysates (cytosol) and Triton X-100 lysates (membrane-bound fraction) were analysed by immunoblotting using antibodies specific for cyt.*c*.

accompanied by the presence of cyt.*c* in the cytosol (lane 2). Release of mitochondrial cyt.*c* was attenuated by glucose, and not detectable in hypoxic cells overexpressing Bcl-2 (lanes 7 and 8). Despite their effects on caspase activation, serine protease inhibitors including TPCK did not prevent the cyt.*c* translocation (lanes 3–6). The immunoblotting results were subsequently confirmed by immunohistochemical localization of cellular cyt.*c* (Figure 8). In control cells, cyt.*c* was present in mitochondria, showing a perinuclear distribution (Figure 8, CON). During hypoxic incubation, cyt.*c* was leaked into the cytosol in some cells, resulting in diffuse staining of the whole cell bodies (Figure 8, 5H). Overexpression of Bcl-2 prevented relocalization of cyt. *c* and maintained the mitochondrial staining (Figure 8, 5H + Bcl-2), as reported previously [13]. In contrast, serine protease inhibitors, including TPCK, did not preserve mitochondrial cyt.*c* during hypoxia (Figure 8, 5H + TPCK).

Suppression of cyt.*c*/dATP-stimulated caspase activation in cytosolic extracts by serine protease inhibitors

Serine protease inhibitors suppressed caspase activation in hypoxic cells but did not prevent release of mitochondrial cyt.*c*, suggesting that a critical step of cyt.*c*-initiated caspase activation was blocked. A key process of cyt.*c*-mediated caspase activation involves assembly of a protein complex called 'apoptosome', which has been identified and characterized by recent studies [26,27]. To determine whether serine protease inhibitors prevent caspase activation at this step, we used an *in vitro* reconstitution model. The results are shown in Figure 9. When added to cytosolic extracts, cyt.*c*/dATP triggered dramatic increases in caspase activity (Figure 9A). Caspase activation stimulated by exogenous cyt.*c*/dATP was attenuated by VAD, as expected. Significantly, preincubation of the cytosolic extracts with TPCK and APF prevented cyt.*c*/dATP-induced caspase activation as well, while TLCK was much less effective at the same concentration (Figure 9A). To further determine the steps blocked by TPCK and APF, we analysed proteolytic processing of the initiator caspases. As shown in Figures 9B and 9C, caspase-9, and not caspase-8, was processed in the presence of cyt.*c*/dATP. Moreover, caspase-9 processing was abolished by VAD, APF and TPCK but not by TLCK. Together, the results suggest that TPCK and APF may block caspase activation at early post-mitochondrial steps required to activate the initiator caspase-9.

DISCUSSION

The current study has examined the proteolytic events responsible for apoptotic cell death during hypoxia–reoxygenation. Caspase-3, -8 and -9 were identified to be processed and activated in hypoxic cells, prior to reoxygenation. Caspase activation was caused by ATP depletion and not by hypoxia *per se*. As expected, caspase activation was abolished by VAD and overexpression of Bcl-2. Of much interest, serine protease inhibitors including TPCK, APF and AEBSF blocked caspase activation as well, without affecting the release of mitochondrial cyt.*c* during hypoxia. These serine protease inhibitors might interfere with the caspase activation complexes or apoptosome, as demonstrated by *in vitro* reconstitution experiments.

Two major pathways of apoptotic signalling have been identified. The first involves ligation of death receptors (e.g. Fas) by their ligands, leading to recruitment of adapter proteins and activation of the initiator, caspase-8 [24]. In the second pathway, mitochondrial cyt.*c* is released into the cytosol and binds Apaf-1, which in turn associates and activates the initiator, caspase-9 [25]. These two separate pathways, triggered in different ways, ultimately converge at the level of executioner caspases which disassemble the committed cells. Our recent studies have demonstrated a role for Bax translocation and mitochondrial cyt.*c* leakage in cell death associated with hypoxia–reoxygenation [13]. Based on these observations, a signalling scheme underlying cell injury by hypoxia–reoxygenation was postulated [13,14]. The present study has identified activation of caspase-3, -8 and -9 during hypoxia, filling the gap between cyt.*c* leakage and apoptosis associated with reoxygenation.

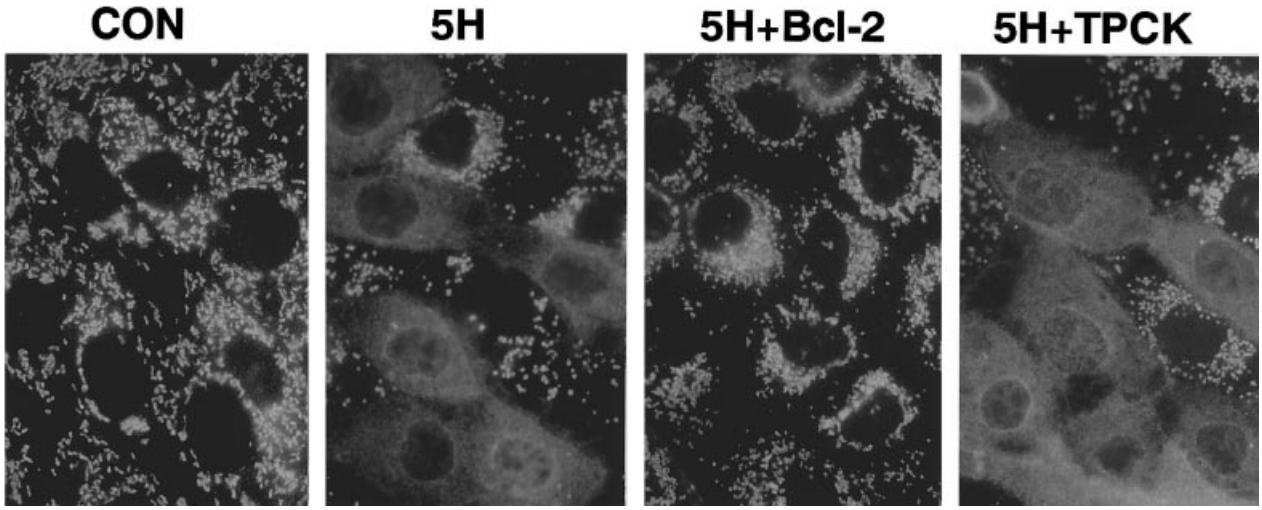

Figure 8 Immunofluorescence of cyt.*c*

Cells (CON: control) were subjected to 5 h of hypoxic incubation in the absence of metabolic substrates without (5H), or with 40 μM TPCK (5H + TPCK). 5H + Bcl-2, 5 h of hypoxia was carried out with cells overexpressing Bcl-2. Following incubation, cells were fixed and processed for immunofluorescence as described in the Materials and methods section.

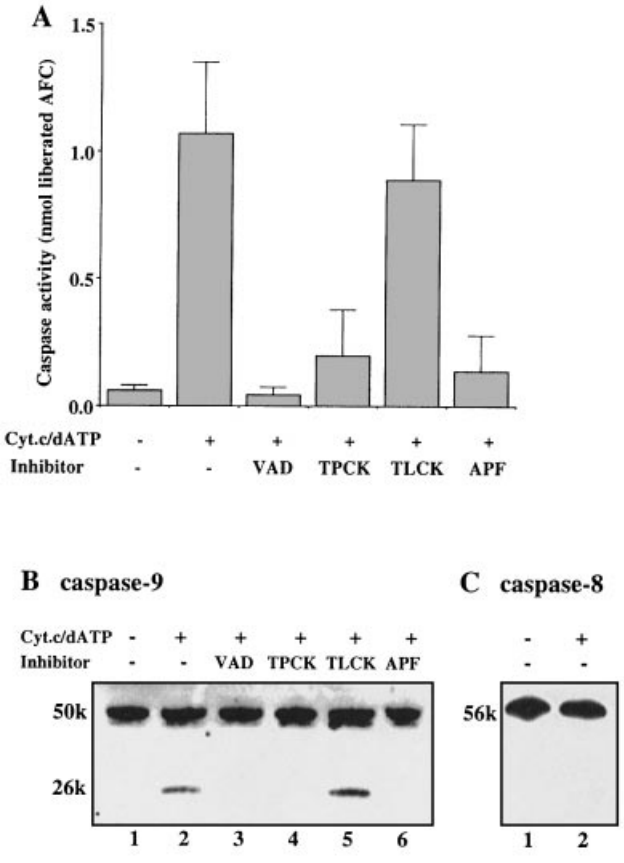

Figure 9 Inhibition of cyt.*c*/dATP-stimulated caspase activation in cytosolic extracts by TPCK and APF

Cells were exposed to 1 h of hypoxia to induce caspase-9 (Figure 1), and cytosol extracted with digitonin as described in the Materials and methods section. Cytosolic extracts were preincubated for 10 min with protease inhibitors (10 μM VAD, 50 μM TPCK, 50 μM TLCK or 50 μM APF) or the solvent DMSO. For reconstitution, 50 μg/ml cyt.*c* and 1 mM dATP were added to the cytosolic extracts and incubated for 1–2 h at 30 or 37 °C. The reconstitution mixtures were finally analysed for DEVDase activity (**A**), caspase-9 activity (**B**) or caspase-8 activity (**C**). Results in **A** are means ± S.E.M. ($n = 4$).

On the basis of a general scheme regarding caspase activation initiated by cyt.*c* [25], it may be postulated that a cascade with caspase-9 at the apex and caspase-3 as the downstream effector drives apoptosis during hypoxia–reoxygenation. However, we were unable to delineate a hierarchy for caspase activation in our studies. Processing and activation kinetics of the different caspases were indistinguishable: undetectable after 3 h of hypoxia, but evident at the end of 5 h of hypoxia (Table 1 and Figure 1, and results not shown). We attempted to examine caspase hierarchy using the selective inhibitors carbobenzoxy-Asp-Glu-Val-Asp-fluoromethyl ketone and carbobenzoxy-Ile-Glu-Thr-Asp-fluoromethyl ketone. These two inhibitors are considered to have specificity for caspase-3 and caspase-8, respectively. However, either inhibitor blocked all caspase activity during hypoxia at 20 μM, and had no effect at 10 μM (results not shown). The results suggested that tight coupling and complexity of caspase activation pose obstacles to the analysis of caspase hierarchies governing apoptotic pathways within intact cells. One explanation is that unknown constraints limit the initial activation of apex caspases below a 'safe' threshold of intensity which can be exceeded only by feedback mechanisms mediated by downstream caspases.

Involvement of caspases in cell injury during hypoxia–reoxygenation has been suggested by other studies showing protective effects of caspase inhibitors [28,29]. These observations could be relevant to cell injury following ischaemia–reperfusion *in vivo*; broad spectrum caspase inhibitors were recently shown to reduce the extent of cell death during reperfusion following ischaemia of the heart and brain [30,31]. However, our current observations suggest that cellular caspase activation may require the participation of non-caspase proteases as well. Thus, proteolytic mechanisms other than those involving caspases could also be potential targets for therapeutic intervention during hypoxic injury. Serine protease inhibitors including TPCK, APF and AEBSF suppressed the activation of caspases in hypoxic cells. Complete inhibition of proteolysis of death substrates and arrest of apoptosis during reoxygenation also resulted. Although the molecular basis underlying the inhibition remains to be identified, these serine protease inhibitors do not target caspases directly, since they could not suppress fully activated caspases in hypoxic cell lysates. Moreover, the inhibitors did not mitigate

mitochondrial release of cyt.*c* during hypoxia. The data suggest that serine proteases may be involved in cyt.*c*-initiated caspase activation and apoptotic commitment. TPCK and APF are classic inhibitors of chymotrypsin-like serine proteases, and the chymotryptic specificity is conferred by the bulky phenylalanine residue. AEBSF, on the other hand, is considered to be a general active-site-directed serine protease inhibitor. Prevention of caspase activation by these compounds but not other protease inhibitors, including TLCK, a tryptic serine protease inhibitor, suggests that the putative protease could be chymotryptic. The lack of effect for PMSF, another general serine protease inhibitor, could be due to its poor solubility and instability in aqueous solutions [22]. DCI was not inhibitory at 10 μM in our experiments, and was highly cytotoxic at higher concentrations.

Participation of serine proteases in apoptosis has been suggested by previous studies using inhibitors [7–12]. More recently, TPCK was shown to be able to induce apoptosis by itself and suppress apoptosis triggered by other stimuli [32,33]. Similar results have been obtained by us with the rat kidney proximal tubule cells (results not shown). Despite these investigations, the steps of apoptotic cascades at which serine protease inhibitors act were not known [5,6]. The experiments that we report here provide firm evidence that TPCK, APF and AEBSF do not prevent cyt.*c* release but inhibit the activation of the initiator caspase-9 during hypoxia. The cyt.*c*-mediated apoptotic pathway involves binding of the cytochrome to the adapter protein Apaf-1, with subsequent incorporation and activation of caspase-9 in a protein complex, apoptosome. Our *in vitro* reconstitution experiments suggest that TPCK and APF interfered with this early step that is required for activating caspase-9. However, involvement of a putative serine protease may well be indirect. Assembly of apoptosome in test-tubes has been achieved with three recombinant molecules: cyt.*c*, caspase-9 and Apaf-1 [26]. Thus, in a pure system, it is not necessary to involve serine proteases. Then, how could the serine protease inhibitors prevent caspase-9 activation in hypoxic cells or the cell cytosol? Our working hypothesis is that activation steps within intact cells are far more complex than a simple combination of three types of molecules. Assembly of apoptosome *in vitro* ensures the availability of free binding sites, proximity and autocatalysis of caspase-9. However, the binding sites *in vivo* may be 'masked' by inhibitory molecules that need to be removed for functional interactions of apoptosomal proteins. As yet to be identified inhibitory molecules, rather than cyt.*c*, caspase-9 or Apaf-1, could be the target of putative serine proteases that may be involved in apoptosome assembly and/or processing.

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