Energy metabolism and protein phosphorylation during apoptosis: a phosphorylation study of tau and high-molecular-weight tau in differentiated PC12 cells*

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Apoptosis has been characterized as a regulated, energy-dependent process. Specific protein-phosphorylation events have been demonstrated previously to occur during apoptosis and may play an important role in the regulation of this death process. In this study, energy metabolism and protein phosphorylation during apoptosis of neuronal PC12 cells induced by nerve growth factor and serum deprivation was examined using [³²P]P_i-labelling techniques. Although ATP levels were maintained at control levels during apoptosis, [³²P]P_i incorporation into ATP was decreased significantly, coinciding with an almost identical decrease in Na⁺-dependent phosphate uptake. During neuronal PC12-cell apoptosis, increased phosphorylation of tau and high-molecular-weight (HMW) tau was observed within the epitope of Tau-1, a phosphate-dependent tau antibody

that only recognizes the unphosphorylated form of its epitope. In addition, based on two-dimensional phosphopeptide mapping, [³²P]P_i incorporation into a phosphopeptide of tau and HMW tau from apoptotic cells increased. Whereas [³²P]P_i incorporation into total protein decreased to 23% of the control during apoptosis, [³²P]P_i incorporation into tau and HMW tau was significantly higher, indicating a preferential phosphorylation of specific proteins during the apoptotic process. This study provides novel information about phosphate uptake, incorporation of [³²P]P_i into ATP, and protein phosphorylation events during apoptosis.

Key words: ATP, cytoskeleton, microtubule-associated protein, peptide mapping, [³²P]P_i labelling.

INTRODUCTION

Apoptosis is a regulated, energy-dependent cell-death process important for a variety of physiological events, including development, tissue homeostasis and cell turnover [1]. Apoptotic cell death has also been implicated in several disease states, such as cancer, and autoimmune and neurodegenerative disorders [2]. Apoptotic cells are morphologically and biochemically defined by cell shrinkage, nuclear and chromatin condensation, and oligonucleosomal DNA fragmentation [3]. For these events to take place during apoptosis, energy levels within the cell must be maintained, as indicated by several studies (e.g. [4,5]). For example, ATP depletion of human T-cells prior to exposure of apoptotic stimuli induces necrosis rather than apoptosis, indicating the importance of cellular ATP levels during the apoptotic process [6]. An ATP-dependent process, protein phosphorylation, may provide a crucial regulatory mechanism during apoptosis.

Protein phosphorylation plays a role in almost every aspect of cell survival, including maintenance, development and division. Whereas the identification of signalling pathways and proteins involved in apoptosis has been the focus of numerous studies, very little is known about the role of phosphorylation in the regulatory processes of apoptotic cell death. However, there are several studies implicating the involvement of protein phosphorylation during apoptosis [7]. An example of this is the activation of c-Jun N-terminal kinase [8], p38 protein kinase [8] and cdc2 kinase [9,10] during apoptosis of various cell lines. The importance of protein phosphorylation during apoptotic cell death is also indicated by the identification of bcl-2 as a phosphoprotein [11] and the implication that phosphorylation of bcl-2 may negatively regulate its anti-apoptotic function [12].

During apoptosis, extensive alterations in the cytoskeleton occur and the phosphorylation of specific cytoskeletal-associated protein may contribute to these structural alterations. One possible target of phosphorylation during apoptosis is the microtubule-associated protein tau. Tau exists as a family of multiple isoforms arising from the same gene [13], and is localized primarily within the axons of neurons in the central nervous system [14]. Tau functions *in vitro* to stabilize microtubules and promote microtubule assembly [15]. The phosphorylation of tau occurs at Ser and Thr residues throughout the molecule, and site-specific phosphorylation negatively regulates the function of tau [16].

Another tau variant, known as high-molecular-weight (HMW) tau, containing exon 4A and/or exon 6 that are not present in tau [17], has been identified in neurons of the peripheral nervous system [18] and in cell lines derived from the neural crest, including PC12 cells [19]. Although it has been hypothesized that HMW tau is similar to tau in terms of function and regulation by phosphorylation, very few studies have been done to support this hypothesis. Differentiated PC12-C41 cells, a subclone of PC12 cells, exhibit increased neurite abundance and stability, and contain a larger proportion of HMW tau when compared with the parental line of differentiated PC12 cells, suggesting different roles of HMW tau and tau in the modulation of neuronal morphology [20]. As a potential microtubule-stabilizing protein, HMW tau may play an important role in response to peripheral-nervous-system nerve injury and, therefore, it is important to investigate its function and regulation by phosphorylation.

In this study, energy metabolism in terms of ATP levels and [³²P]P_i incorporation into intracellular ATP pools was examined during apoptosis of neuronal PC12 cells induced by removal of

Abbreviations used: HMW, high molecular weight; NGF, nerve growth factor; PP2B, protein phosphatase 2B.

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trophic support. Apoptosis of neuronal PC12 cells induced by trophic-factor deprivation is well established [21,22]. Like other laboratories, our laboratory has demonstrated that PC12 cells deprived of serum and nerve growth factor (NGF) undergo apoptosis, exhibiting decreased viability as indicated by Trypan Blue exclusion and lactate dehydrogenase release and also showing chromatin condensation as indicated by Acridine Orange staining in a time-dependent manner [10]. Differentiated PC12 cells deprived of serum and NGF for 48 h exhibit a significant decrease (approx. 30%) in viability when compared with control cells maintained in serum-free medium with NGF [10]. At this time point, 24% of the serum- and NGF-deprived cells exhibit a very bright punctate Acridine Orange staining pattern of chromatin, indicating condensation. Additionally, cells deprived of serum and NGF are reduced in size when compared with control cells. After 72 h of serum and NGF deprivation, 52% of differentiated PC12 cells were apoptotic when determined by chromatin condensation and cell viability [10]. Over the entire time course, control cells maintained in serum-free media in the presence of NGF remain viable and exhibit a diffuse granular chromatin-staining pattern [10].

Although ATP levels did not decrease significantly during PC12 cell apoptosis, [^{32}P]P_i incorporation into ATP did decrease significantly, which coincided with a similar decrease in Na⁺-dependent [^{32}P]P_i transport into the cell. To our knowledge, this is the first study of [^{32}P]P_i metabolic labelling in apoptotic cells. Additionally, phosphorylation of tau and HMW tau during apoptosis of PC12 cells was investigated using immunoblot analysis and [^{32}P]P_i metabolic labelling. Phosphorylation within the Tau-1 epitope in both tau and HMW tau increased significantly during apoptosis. Whereas tau protein levels decreased during PC12-cell apoptosis, HMW tau protein levels were not altered significantly, suggesting differential regulation and roles of tau and HMW tau. The novel findings of this study with regards to [^{32}P]P_i transport and incorporation into ATP during apoptosis provides important information for future studies of protein-phosphorylation events during apoptotic cell death.

MATERIALS AND METHODS

Cell culture

PC12 cells were grown on rat tail collagen-coated dishes in RPMI 1640 media containing 10% horse serum, 5% Fetal Clone II (Hyclone, Logan, UT, U.S.A.), 20 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, as described previously [23]. For neuronal differentiation, PC12 cells were maintained in media containing 5% serum and 100 ng/ml NGF for 9–10 days [23]. To remove serum and NGF, differentiated PC12 cells were replated on to new collagen-coated dishes as described previously [10]. After replating, PC12 cells were maintained in serum-free media with or without 100 ng/ml NGF for the times indicated.

Determination of intracellular ATP and ADP levels

Intracellular levels of ATP and ADP were determined using the luciferase/luciferin assay system [24]. Cells maintained on 35-mm dishes were rinsed once and removed from the dish with PBS [1.5 mM KH₂PO₄/8.0 mM Na₂HPO₄/2.7 mM KCl/137 mM NaCl (pH 7.4)]. After a brief centrifugation, the cells were resuspended in lysis buffer containing 70 mM Tris/HCl (pH 7.75), 2 mM EDTA, 60 µM dithiothreitol and 0.2% Triton X-100, placed in a dry-ice/methanol bath, and stored at -80 °C until use. Protein concentrations were determined by using the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.). Cell

homogenate containing 1–2 mg/ml protein was added to a mixture containing 0.063% (w/v) BSA, 10 mM magnesium acetate and 100 mM Tris/HCl (pH 7.75). The reaction was initiated by the addition of 70 µM luciferin and 0.05 µg/ml luciferase, and luminescence was measured using a scintillation counter with the coincidence gate inactivated. ADP was converted to ATP by the addition of 2 units/mg pyruvate kinase/5 mM phosphoenolpyruvate to the reaction mixture 20 min prior to the addition of luciferase and luciferin [25]. ADP levels were calculated as the difference between counts from the reactions with and without pyruvate kinase and phosphoenolpyruvate. The amount (nmol) of ATP was determined using an ATP standard curve.

Immunoblotting and alkaline phosphatase treatment

Cell homogenates were prepared for immunoblotting as described previously [10]. Following acid precipitation, protein was determined by the method of Lowry et al. [26]. Equal amounts of protein were separated by SDS/PAGE (7.5% gel), transferred on to nitrocellulose, and probed with monoclonal tau antibodies Tau-1 or Tau-5. The phosphate-dependent antibody Tau-1 recognizes tau or HMW tau only when Ser¹⁹⁵, Ser¹⁹⁸, Ser¹⁹⁹, Ser²⁰² and Thr²⁰⁵ (numbered according to the longest human brain tau isoform [27]) are *not* phosphorylated [14,28]. To determine protein levels of tau and HMW tau, the phosphate-independent Tau-5 antibody [29] was used. After incubation with horseradish peroxidase-conjugated secondary antibody, immunoblots were developed using 3,3'-diaminobenzidine. Immunoblots were quantified using a BioRad GS-670 imaging densitometer. Alkaline phosphatase treatment of blots prior to incubation with antibody was done as described previously [30]. Blots incubated in the presence or absence of alkaline phosphatase treatment were compared by internal normalization with a known amount of the longest isoform of human brain tau (T4L), which was run on each blot. T4L was expressed in bacteria and purified as described previously [31].

[^{32}P]P_i metabolic labelling and immunoprecipitation

For [^{32}P]P_i labelling *in situ*, PC12 cells were rinsed once with incubation medium (phosphate-deficient RPMI 1640 containing 20 mM glutamine, 0.1 µg/ml transferrin and 15 nM selenious acid). Cells were preincubated in incubation medium for 30 min and labelled subsequently with 0.6 mCi/ml [^{32}P]P_i (ICN, Costa Mesa, CA, U.S.A.) for 4 h at 37 °C. After labelling, cells were rinsed once with PBS and scraped into extraction buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.2% Triton X-100/2 mM EDTA/2 mM EGTA/10 µg/ml leupeptin/10 µg/ml aprotinin/5 µg/ml pepstatin A/100 µM PMSF/0.1 µM okadaic acid]. Extracts were clarified by brief centrifugation at 4 °C, and protein concentrations were determined by the bicinchoninic acid assay. To determine [^{32}P]P_i incorporation into total protein, samples from control and apoptotic cells containing 2 µg of protein with the addition of 8 µg of BSA were precipitated using trichloroacetic acid. [^{32}P]P_i incorporation into the precipitated protein was determined by scintillation counting. The remaining protein of each sample was used for tau and HMW tau immunoprecipitation and phosphopeptide mapping.

Equivalent amounts of protein were incubated with Tau-5 conjugated to Actigel ALD (Sterogene, Carlsbad, CA, U.S.A.) for 2 h at 4 °C. Following incubation, the immune complexes were washed twice each with 0.4% Nonidet P-40/PBS, 0.1% Tween-20/PBS and PBS. Immune complexes were resuspended in 2 × Laemmli sample buffer without reducing agents and boiled for 5 min. Dithiothreitol was added to the supernatants con-

Figure 1 Changes in the protein levels and phosphorylation of tau and HMW tau at the Tau-1 epitope from control (+) and NGF-deprived (–) PC12 cells

After serum and/or NGF deprivation for the times indicated, cell homogenates were prepared, and equivalent amounts of total protein were separated by SDS/PAGE (7.5% gel). Tau and HMW tau protein levels were determined by immunoblotting with a phosphate-independent antibody, Tau-5, (A), and phosphorylation changes of tau and HMW tau were determined by immunoblotting with Tau-1 (B), which only binds when Ser residues within its epitope are unphosphorylated. Therefore, a decrease in immunoreactivity indicates an increase in tau phosphorylation at the Tau-1 epitope. Immunoblots are representative of at least three

taining immunoprecipitated protein at a final concentration of 5 mM, and the samples were boiled for 5 min. The samples were electrophoresed on SDS/7.5% polyacrylamide gels and immunoblotted as described above using the Tau-5 antibody. To determine [32 P] P_i incorporation into tau, immunoblots were analysed using the PhosphorImager System with ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). [32 P] P_i incorporation into tau or HMW tau was normalized to the amount of tau or HMW tau protein present in each sample, as determined by quantitative densitometric analysis of Tau-5 immunoreactivity.

For two-dimensional phosphopeptide mapping, HMW tau was immunoprecipitated using a monoclonal antibody that recognizes an epitope within exon 4A that is present only in HMW tau [32]. Cell extracts were prepared as described above and preincubated with protein A-Sepharose beads for 30 min at 4 °C to reduce non-specific binding. Pre-cleared samples were incubated with the exon 4A antibody for 1 h followed by another 1 h incubation with protein A-Sepharose beads at 4 °C. Immune complexes were washed five times with extraction buffer and three times with 50 mM Tris/HCl (pH 7.5), then processed for two-dimensional phosphopeptide mapping as described below.

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis

To enrich tau protein, a heat-stable/perchloric acid-soluble fraction was prepared from 32 P-labelled cell extracts according to Fleming and Johnson [33]. The samples were separated on a SDS/7.5% polyacrylamide gel, stained with Coomassie Brilliant Blue R-250, destained and washed extensively with 20% methanol to remove residual acetic acid. Tau bands were excised from the gel, and the gel pieces were frozen and lyophilized. Immune complexes with HMW tau or gel pieces containing tau were treated sequentially with *N*- α -tosyl-L-lysine-chloromethyl-ketone-treated α -chymotrypsin and tosyl-L-phenylalanine-chloromethyl-ketone-treated trypsin, as described previously [33]. Two-dimensional phosphopeptide mapping was carried out as described by Sihag and Nixon [34]. Phosphopeptide maps were analysed with the PhosphorImager System and ImageQuant analysis software. Phosphoamino acid analysis of the phosphopeptide designated 'a' was carried out exactly as described by van der Geer et al. [35].

[γ - 32 P]ATP specific activity

[32 P] P_i at a concentration of 0.012 mCi/ml was used to label PC12 cells as described above. Following incubation, cells were rinsed once with PBS, and nucleotides were extracted from cells with 1 M formic acid and purified by reversed-phase HPLC as described by Payne and Ames [36]. Briefly, nucleotides were separated on a C_{18} column (25 cm $\times$ 4.6 mm) using solvent I, consisting of 5 mM tetrabutylammonium phosphate, 4% acetonitrile and 30 mM KH_2PO_4 (pH 6.0), and solvent II, consisting of 80% acetonitrile. Nucleotides were allowed to elute using a

independent experiments. The positions at which molecular mass markers (kDa) migrated are indicated to the left. Quantitative analysis of alterations in tau (●) and HMW tau (■) protein levels (C) and phosphorylation at the Tau-1 epitope (D) in response to serum-free (SF) conditions without NGF are expressed as a percentage of control values (maintained in SF conditions with NGF, SF + NGF). Tau-1 immunoblot data are presented as the levels of phosphorylation within the Tau-1 epitope of tau and HMW tau after normalization to changes in tau and HMW tau protein levels during apoptosis. The quantified data represent the means $\pm$ S.E.M. of at least three independent experiments. * $P \leq 0.05$ when compared with control values (SF + NGF).

solvent I–solvent II gradient of 0–50% over 40 min with a 1 ml/min flow rate. Typical retention times for AMP, ADP and ATP were 12.7, 20.0 and 27.5 min, respectively, as determined by the use of standards (Sigma, St. Louis, MO, U.S.A.). To circumvent variability between HPLC applications, 85 μ M dideoxyinosine was added to each sample as an internal standard, and the dideoxyinosine peak area was used for normalization. ATP (in nmol) in each sample was determined using a standard ATP curve, and [32 P] P_i incorporation into ATP was determined by scintillation counting. [32 P] P_i incorporation into the γ -phosphate position of ATP was examined as described by Cheetham and Lardy [37]. Briefly, 100 μ l of the [32 P]ATP fraction from each sample was added to a reaction mixture containing 6 units/ml hexokinase, 100 μ M ATP, 1 mM glucose, and 40 mM Tris/HCl (pH 7.5). The reaction mixture was incubated for 2.5 h at room temperature, and the reaction was stopped by brief incubation of the tube in a dry-ice/methanol bath and subsequent lyophilization. Samples were re-applied to the column, and AMP, ADP and ATP fractions were collected. The d.p.m. value of each fraction was determined by scintillation counting. The [32 P] P_i incorporation into the γ -phosphate position of ATP was determined by subtracting the total d.p.m. value of the ADP and AMP fractions from the total d.p.m. input.

Na⁺-dependent and -independent phosphate transport into PC12 cells

[32 P] P_i transport into the cell was measured as described by Furman et al. [38] with one modification. Cells were phosphate-starved by preincubation with phosphate-deficient media, as described above, prior to the assay.

RESULTS

Maintenance of ATP during differentiated PC12 cell apoptosis

Previously, studies have demonstrated that ATP levels are maintained during apoptosis [4]. To determine the metabolic state of the cell, ATP and ADP levels were measured in control and apoptotic PC12 cells at different times after serum and/or NGF removal. ATP levels from PC12 cells deprived of serum and NGF remained at control levels throughout the 72 h time period examined. After 72 h there were 8.3 ± 1.9 nmol of ATP/mg

of protein in control cells and 11.0 ± 2.1 nmol of ATP/mg of protein in cells deprived of serum and NGF ($n = 3$ separate experiments). Additionally, ADP levels were not altered significantly in differentiated PC12 cells deprived of serum and NGF for 48 h (control, 3.6 ± 1.7 nmol of ADP/mg of protein; apoptotic, 4.0 ± 0.5 nmol of ADP/mg of protein, $n = 3$). These results strongly suggest that ATP-dependent processes such as phosphorylation still occur during apoptosis.

Increased phosphorylation within the Tau-1 epitope of tau and HMW tau during apoptosis

Initially, the phosphorylation of tau and HMW tau from PC12 cells was examined at various times throughout serum and NGF deprivation by immunoblot analysis. The Tau-1 monoclonal antibody that recognizes an *unphosphorylated* epitope was used to examine alterations in tau and HMW tau phosphorylation during apoptosis. Because alterations in tau and HMW tau protein levels may occur during apoptosis, tau and HMW tau protein levels from control and apoptotic PC12 cells were examined by immunoblotting with the phosphate-independent tau antibody, Tau-5 (Figure 1A). Tau-1 immunoreactivity towards tau and HMW tau decreased in PC12 cells deprived of serum and NGF when compared with the control, thus indicating an increase in tau and HMW tau phosphorylation within the Tau-1 epitope (Figure 1B).

Figures 1(C) and 1(D) show quantified Tau-5 and Tau-1 immunoblot data, respectively, for tau and HMW tau. Tau protein levels, as indicated by the amount of Tau-5 immunoreactivity, significantly decreased by 34% in PC12 cells after 48 h of serum and NGF deprivation when compared with control tau protein levels (Figure 1C). Following 72 h of serum and NGF deprivation, tau protein levels in apoptotic PC12 cells decreased by 42% compared with control (Figure 1C). In contrast to tau protein levels, HMW tau protein levels did not decrease significantly during apoptotic PC12 cell death over the time period examined (Figure 1C).

When normalized to tau protein levels, the phosphorylation of tau within the Tau-1 epitope increased significantly by 35 and 21% from cells deprived of serum and NGF for 48 and 72 h, respectively, when compared with control (Figure 1D). As early as 32 h following serum and NGF deprivation, Tau-1

Figure 2 Recovery of Tau-1 immunoreactivity following treatment with alkaline phosphatase

Blots containing equivalent amounts of total protein from control (+) or apoptotic (−) PC12 cells following 48 or 72 h of serum and NGF deprivation were incubated in the presence (+, right-hand panel) or absence (−, left-hand panel) of alkaline phosphatase and subsequently probed with Tau-1. The positions at which molecular-mass markers (kDa) migrated are indicated to the left. The alkaline phosphatase-induced increase in Tau-1 immunoreactivity of tau and HMW tau from apoptotic PC12 cells was greater when compared with controls, demonstrating an increased phosphorylation within the Tau-1 epitope of tau and HMW tau during PC12 cell apoptosis. Immunoblots are representative of three separate experiments.

Figure 3 Alterations in [^{32}P]P $_i$ incorporation into ATP and Na $^+$ -dependent [^{32}P]P $_i$ uptake during PC12 cell apoptosis

Differentiated PC12 cells were maintained in serum-free medium with (black bars) or without (hatched bars) NGF for 48 h. [^{32}P]P $_i$ incorporation into cellular ATP was measured, and the data are expressed as d.p.m./pmol of ATP (A). Na $^+$ -dependent and -independent [^{32}P]P $_i$ uptake into the cell was determined and expressed as nmol of [^{32}P]P $_i$ /mg of protein per min (B). Values indicate the means $\pm$ S.E.M., $n \geq 3$. * $P \leq 0.05$ indicates values that are significantly different when compared with control (with NGF) values.

phosphorylation of HMW tau from apoptotic PC12 cells was significantly increased when compared with controls (Figure 1D). During the apoptotic process, phosphorylation of the Tau-1 epitope in HMW tau continued to increase such that at 72 h of serum and NGF deprivation, Tau-1 phosphorylation of HMW tau significantly increased by 50 % when compared with controls (Figure 1D).

Recovery of Tau-1 immunoreactivity towards tau and HMW tau from apoptotic PC12 cells following alkaline phosphatase treatment

To confirm that the significant decrease in Tau-1 immunoreactivity towards tau and HMW tau from apoptotic PC12 cells

Table 1 [^{32}P]P $_i$ incorporation into tau and HMW tau was increased significantly compared with [^{32}P]P $_i$ incorporation into total protein from PC12 cells deprived of serum and NGF for 48 h

[^{32}P]P $_i$ -labelled tau and HMW tau were immunoprecipitated from control and apoptotic PC12 cells after 48 h of serum and NGF deprivation, and the obtained protein levels from control and apoptotic PC12 cells used to normalize [^{32}P]P $_i$ incorporation into tau and HMW tau as determined by PhosphorImager Analysis of the immunoblots. [^{32}P]P $_i$ incorporation into total protein was determined using equivalent amounts of total protein from control and apoptotic PC12 cells, and the data were also normalized to account for the difference in [^{32}P]ATP specific activity between control and apoptotic cells. Data are expressed as a percentage of control (serum-free conditions with NGF) and represent the means $\pm$ S.E.M. * $P \leq 0.05$ indicates a significant increase in [^{32}P]P $_i$ incorporation into tau and HMW tau compared with [^{32}P]P $_i$ incorporation into total protein.

	[^{32}P]P $_i$ incorporation	[^{32}P]P $_i$ incorporation normalized to [^{32}P]ATP
Tau	50.60 $\pm$ 7.40*	70.30 $\pm$ 2.50*
HMW tau	41.71 $\pm$ 6.00*	57.90 $\pm$ 5.10*
Total protein	23.80 $\pm$ 1.93	33.10 $\pm$ 4.70

was due to the presence of phosphorylated residues within the Tau-1 epitope, blots containing tau and HMW tau from control or apoptotic PC12 cells were incubated in the absence or presence of alkaline phosphatase, and alterations in Tau-1 immunoreactivity were examined. Representative immunoblots of these experiments are shown in Figure 2. Treatment of immunoblots with alkaline phosphatase resulted in increased Tau-1 immunoreactivity, which is indicative of a decrease in phosphorylation within the epitope, for both control and apoptotic samples. However, the alkaline phosphatase-induced increase in Tau-1 immunoreactivity was significantly greater for tau and HMW tau from apoptotic cells when compared with controls (Figure 2). Alkaline phosphatase-induced increases in Tau-1 immunoreactivity of tau and HMW tau from apoptotic PC12 cells deprived of serum and NGF for 48 h were 4.9- and 3.3-fold greater, respectively, than the alkaline phosphatase-induced increases in Tau-1 immunoreactivity of tau and HMW tau from control cells. Similar results were also observed for tau and HMW tau from apoptotic PC12 cells deprived of serum and NGF for 72 h ($n = 3$ separate experiments). The results demonstrate an increase in the phosphorylation of tau and HMW tau within the Tau-1 epitope during PC12 cell apoptosis.

A decrease in phosphate uptake into apoptotic PC12 cells and its effect on [^{32}P]ATP specific activity

Because phosphorylation within the Tau-1 epitope increased significantly in tau and HMW tau from PC12 cells beginning at 48 h of serum and NGF deprivation, further experiments involving [^{32}P]P $_i$ metabolic labelling were carried out at this time point. Since [^{32}P]P $_i$ incorporation into tau and HMW tau may be affected by differences in [^{32}P]ATP specific activity between control and apoptotic PC12 cells, [^{32}P]P $_i$ incorporation into cellular ATP was determined 48 h after serum and NGF removal. [^{32}P]P $_i$ incorporation into ATP from apoptotic PC12 cells after 48 h of serum and NGF deprivation decreased significantly by 38.9 % compared with controls (Figure 3A). Although total [^{32}P]P $_i$ incorporation into ATP decreased significantly during apoptosis, the percentage of [^{32}P]P $_i$ in the γ -phosphate position of ATP relative to total [^{32}P]P $_i$ in ATP did not change between control and apoptotic PC12 cells (results not shown).

Figure 4 Representative two-dimensional phosphopeptide maps of tau and HMW tau from control and apoptotic PC12 cells

[^{32}P] P_i -labelled tau or HMW tau from control or apoptotic PC12 cells after 48 h of serum and NGF removal were subjected to two-dimensional phosphopeptide mapping. Equivalent amounts of d.p.m.s were applied to each plate. Phosphopeptide maps of tau from control [serum-free (SF) conditions with NGF, SF + NGF] (A) and apoptotic (SF without NGF, SF - NGF) (B) cells, and HMW tau from control (C) and apoptotic (D) cells are shown and are representative of the results obtained in three independent experiments. Arrowheads indicate phosphopeptides as reference points for comparison between maps. [^{32}P] P_i incorporation into tau and HMW tau phosphopeptide **a** from apoptotic PC12 cells when normalized internally to phosphopeptide **b** is significantly increased compared with control cells. Relative arbitrary units as determined by ImageQuant analysis of the indicated phosphopeptides are given in parentheses: SF + NGF, tau, **a** (55.4) and **b** (48.2); SF - NGF, tau, **a** (183.2) and **b** (35.2); SF + NGF, HMW tau, **a** (74.3) and **b** (26.8); and SF - NGF, HMW tau, **a** (197.5) and **b** (22.3). Some decreased resolution of peptide maps occurred during file conversion from the ImageQuant format to a graphic format and, therefore, the representative phosphopeptide maps shown are not exact replicas of the original data.

One possible reason for the significant decrease in [^{32}P] P_i incorporation into ATP during apoptotic cell death is that phosphate transport into the cell may be compromised. Previously, it has been demonstrated that differentiated PC12 cells have an Na^+ - P_i transporter, which is responsible for the majority of phosphate uptake [38]. Therefore, Na^+ -dependent and independent P_i transport into control and apoptotic PC12 cells was measured. Na^+ -independent mechanisms of P_i uptake into the cell did not differ significantly between control and apoptotic PC12 cells (Figure 3B). In contrast, Na^+ -dependent P_i uptake into apoptotic PC12 cells was decreased significantly compared with control cells (Figure 3B). The 37% decrease in Na^+ -dependent P_i uptake parallels the decrease in [^{32}P] P_i incorporation into ATP during apoptosis (Figure 3B). This finding strongly suggests that a compromised Na^+ -dependent P_i -uptake system may be largely responsible for the decrease in [^{32}P] P_i incorporation into ATP.

[^{32}P] P_i incorporation into tau and HMW tau is increased compared with [^{32}P] P_i incorporation into total protein during apoptosis

To determine the extent of overall phosphorylation of tau and HMW tau from control and apoptotic PC12 cells, [^{32}P] P_i incorporation into tau and HMW tau was measured. Additionally, [^{32}P] P_i incorporation into total protein was measured to determine the extent of total protein phosphorylation occurring in control and apoptotic PC12 cells.

[^{32}P] P_i incorporation into total protein from apoptotic cells was only 23% of control (Table 1). Although [^{32}P] P_i incorporation into tau and HMW tau from apoptotic PC12 cells was not increased compared with control, it was significantly increased over [^{32}P] P_i incorporation into total protein (Table 1). When the data were normalized to the decrease in [^{32}P] P_i specific activity, the percentage of control values increased;

however, [^{32}P]P_i incorporation into tau, HMW tau and total protein from apoptotic and control PC12 cells was not altered (Table 1).

Phosphopeptide mapping of tau and HMW tau from control and apoptotic PC12 cells

Although the overall phosphorylation of tau and HMW tau during apoptosis did not increase compared with control, the phosphorylation of specific residues in tau and HMW tau may be increased in apoptotic PC12 cell death. To determine peptide-specific phosphorylation differences between control and apoptotic PC12 cells, two-dimensional phosphopeptide mapping of tau and HMW tau was carried out.

Figure 4 shows representative phosphopeptide maps of tau and HMW tau from control and apoptotic PC12 cells after 48 h of serum and/or NGF deprivation. [^{32}P]P_i incorporation into phosphopeptide **b** from control and apoptotic PC12 cells was similar based on the results of three independent experiments (HMW tau, 82% of control; tau, 90% of control). Thus phosphopeptides were normalized internally to phosphopeptide **b** for comparison of phosphopeptide maps between control and apoptotic conditions. Although tau and HMW tau phosphopeptide maps appeared similar, comparisons between them could not be made due to possible differences in the resulting proteolytic digests that could occur because of the additional exon(s) present in HMW tau.

In the tau phosphopeptide maps, [^{32}P]P_i incorporation into phosphopeptide **a** from apoptotic PC12 cells increased 2.2-fold over the control (Figure 4). Similarly, [^{32}P]P_i incorporation during apoptosis into phosphopeptide **a** from HMW tau increased 1.9-fold over the control (Figure 4). These findings demonstrate that even though a total increase in phosphorylation of tau and HMW tau was not observed during apoptosis, specific tau and HMW tau peptides were phosphorylated to a greater extent than tau and HMW tau from control, which may be very important for regulating tau during the apoptotic process. To determine the phosphoamino acids present in phosphopeptide **a** of tau, phosphoamino acid analysis was carried out. Phosphopeptide **a** of tau contained both phosphoserine and phosphothreonine residues, indicating the presence of at least two distinct phosphorylation sites within this phosphopeptide (results not shown).

DISCUSSION

The importance of protein-phosphorylation events during apoptosis has been demonstrated by numerous studies. For example, the activities of several protein kinases have been shown to increase [8,9] and the protein levels and activities of protein phosphatases decrease [10]. Thus far, the study of protein phosphorylation during apoptosis has been carried out primarily by using enzyme activity measurements *in vitro* [9,10] and immunoblot analysis [39]. In one study, thymocytes were labelled with [^{32}P]P_i prior to treatment with gliotoxin, which induced apoptosis within 1 h and resulted in the phosphorylation of histone H3 [40].

This is the first study to examine ATP metabolism using [^{32}P]P_i during the apoptotic process and provides novel information regarding protein-phosphorylation processes during apoptosis. The findings of this study were: (i) whereas ATP levels are maintained, [γ - ^{32}P]ATP specific activity is reduced significantly in apoptotic PC12 cells compared with control cells; (ii) Na⁺-dependent phosphate uptake is decreased significantly during apoptosis of PC12 cells; (iii) during apoptosis, tau protein levels

are significantly decreased while HMW tau protein levels remain at control levels; (iv) as indicated by immunoblot analysis, the phosphorylation of tau and HMW tau within the Tau-1 epitope is significantly increased during apoptosis of PC12 cells; (v) although the overall phosphorylation of tau and HMW tau is not observed, [^{32}P]P_i incorporation into tau is significantly greater than [^{32}P]P_i incorporation into total protein during apoptosis; and (vi) [^{32}P]P_i incorporation into a phosphopeptide of tau and HMW tau from apoptotic PC12 cells is increased compared with control cells.

Similar to a previous study of apoptotic neuronal PC6-3 cells, a subclone of PC12 cells [4], ATP levels remained near control levels during apoptosis of differentiated PC12 cells. However, in this previous study phosphate incorporation into ATP was not examined. To conduct phosphorylation studies using [^{32}P]P_i, the levels of [^{32}P]P_i incorporation into ATP must be equivalent between control and experimental conditions [41] and, therefore, determining [^{32}P]P_i incorporation into ATP was critical for this study. The significant decrease in [γ - ^{32}P]ATP specific activity during differentiated PC12 cell apoptosis is most likely due to the decrease in Na⁺-dependent phosphate uptake into apoptotic PC12 cells. Previous studies have shown that Na⁺-dependent phosphate uptake is responsible for over 90% of saturable phosphate uptake in rat cortical neurons, and that one role of this Na⁺-dependent transporter is to supply phosphate for ATP biosynthesis [42]. Intracellular phosphate levels are dictated by the balance of the ATP production and utilization as well as the flux of phosphate between intracellular and extracellular environments [43]. Whereas a significant decrease in [^{32}P]P_i incorporation into ATP during apoptosis of differentiated PC12 cells was observed, it does not necessarily indicate a decrease in ATP biosynthesis. An increase in the utilization of other intracellular sources of phosphate for ATP biosynthesis may be occurring during apoptosis to maintain ATP levels. Conversely, ATP utilization may be decreased in apoptotic cells, and therefore a decrease in ATP biosynthesis would not result in overall decreases in ATP levels during apoptotic cell death.

Tau and HMW tau may play a role in the cytoskeletal reorganization that occurs during neuronal apoptotic cell death. HMW tau protein levels remain at control levels while tau protein levels significantly decrease during differentiated PC12 cell apoptosis. This result suggests differential functions of HMW tau and tau during apoptotic death of neuronal PC12 cells. Since tau and HMW tau arise from the same gene, differential expression of tau and HMW tau protein may be regulated by mRNA stability or by factors controlling alternative mRNA splicing. Additionally, differential susceptibility of tau and HMW tau to proteolysis may account for differences in tau and HMW tau protein levels during apoptosis. Further investigation of HMW tau and tau cellular localization and microtubule-association in control and apoptotic differentiated PC12 cells should provide information regarding their function during apoptotic PC12 cell death.

Site-specific phosphorylation changes in tau and HMW tau may alter their localization and function and hence contribute to the extensive cytoskeletal changes that occur during apoptosis. Immunoblot analysis revealed an increase in tau and HMW tau phosphorylation within the Tau-1 epitope during apoptosis. Whereas increased phosphorylation within the Tau-1 epitope of tau and HMW tau during apoptosis was observed, hyperphosphorylation of other serine and/or threonine residues of tau and HMW tau during apoptosis cannot be ruled out. In apoptotic PC12 cells, there was a significant increase in [^{32}P]P_i incorporation into tau and HMW tau compared with [^{32}P]P_i incorporation into total protein. Although [^{32}P]P_i incorporation into tau and HMW

tau increased relative to total protein, no overall increase in [^{32}P]P_i labelling of tau and HMW tau occurred during apoptosis. In tau and HMW tau, ^{32}P phosphorylation of a specific phosphopeptide was greatly increased compared with control. Additionally, the presence of both phosphoserine and phosphothreonine within this phosphopeptide of tau suggests the possibility of multiple hyperphosphorylated sites. These data taken together suggest that during apoptosis the phosphate turnover at specific residues is differentially altered with the net effect resulting in no overall increase in [^{32}P]P_i labelling of tau and HMW tau. However, the fact that [^{32}P]P_i incorporation into tau and HMW tau was significantly increased compared with total protein suggests that phosphorylation of tau and HMW tau may lead to alterations of tau and HMW tau function during the apoptotic process.

The increase in Tau-1 phosphorylation and the increase in the phosphorylation of a phosphopeptide from tau and HMW tau during differentiated PC12 cell apoptosis may be due to an increase in protein kinase activity, a decrease in protein phosphatase activity or both. An increase in the activity of cdc2 protein kinase [10,44] and c-Jun N-terminal protein kinase [8] has been demonstrated during differentiated PC12 cell apoptosis. cdc2 protein kinase [45] phosphorylates tau *in vitro* at the Tau-1 epitope and could contribute to the increase of tau and HMW tau phosphorylation at this site during apoptosis of differentiated PC12 cells. Furthermore, protein phosphatase 2B (PP2B, also known as calcineurin) activity is decreased in apoptotic differentiated PC12 cells [10], and tau is dephosphorylated *in vitro* by this phosphatase, resulting in increased Tau-1 immunoreactivity [46]. Additionally, tau has been identified as an *in situ* substrate of PP2B [28]. Therefore, a decrease in PP2B activity during apoptosis could contribute to the increased phosphorylation within the Tau-1 epitope. Recently, it was reported that AT8 immunocytochemical staining of apoptotic PC12 cells was increased compared with controls [47]. This is intriguing because AT8 recognizes tau only when it is phosphorylated at Ser²⁰² and Thr²⁰⁵, which are located within the Tau-1 epitope [48], and hence these data support the findings of the current study.

Extensive tau hyperphosphorylation occurs within the dying neurons in Alzheimer's disease brain [49]. Intriguingly, tau hyperphosphorylation within the Tau-1 epitope was one of the earliest tau phosphorylation changes observed in Alzheimer's disease [50]. The further study of mechanisms involving tau hyperphosphorylation within the Tau-1 epitope during PC12 cell apoptosis may provide information regarding mechanisms responsible for tau hyperphosphorylation in Alzheimer's disease.

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