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Effects of age and calorie restriction on tryptophan nitration, protein content and activity of succinyl-CoA:3-ketoacid CoA transferase in rat kidney mitochondria

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Abstract

This study examined the protein targets of nitration and the consequent impact on protein function in the rat kidney mitochondria at 4-, 13-, 19- and 24-months of age. Succinyl-CoA transferase (SCOT), a rate-limiting enzyme in the degradation of ketone bodies was the most intensely reactive protein against anti-3-nitrotyrosine antibody in the rat kidney mitochondria. However, subsequent mass spectrometric and amino acid analysis of purified SCOT indicated that tryptophan 372, rather than a tyrosine residue, was the actual site of simultaneous additions of nitro and hydroxy groups. This finding suggests that identification of nitrated tyrosine residues, solely based on reactivity with anti-3-nitrotyrosine antibody, can be potentially misleading. Between 4 and 24 months of age, the amount of SCOT protein and catalytic activity, expressed as per milligram mitochondrial proteins, decreased by 55% and 45%, respectively. SCOT and particularly its nitrated carboxy-terminal region were relatively more susceptible to *in vitro* proteolysis than other randomly selected kidney mitochondrial proteins. The age-related decreases in SCOT protein amount and catalytic activity were prevented by a relatively long-term 40% reduction in the amount of food intake. Loss of SCOT protein in the aged rats may attenuate the capacity of kidney mitochondria to utilize ketone bodies for energy production.

Keywords

protein nitration; tryptophan nitration; succinyl-CoA transferase; aging; caloric restriction; mitochondrial enzymes

Introduction

Specific proteins become increasingly nitrated during the aging process or under certain pathological conditions [1–11]. It is widely supposed that protein nitration occurs predominantly via the addition of a nitro group (-NO_2) onto the aromatic amino acid residue,

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tyrosine [12,13]. In most instances, this inference has been based solely on a positive reaction between the target protein and the anti-3-nitrotyrosine (3NT) antibody. However, a recent study in this laboratory indicated that this antibody could also react with nitrated tryptophan residues. Specifically, using anti-3NT antibody, succinyl-CoA:3-ketoacid CoA transferase (SCOT), an intra-mitochondrial, rate-limiting enzyme in the degradation of ketone bodies, was found to be the most prominent target of nitration in the rat heart. However, subsequent mass spectrometric and amino acid analyses indicated that the affected residue was tryptophan 372 with a nitro (-NO_2) and a hydroxy (-OH) adduct, rather than a tyrosine residue [14].

The physiological issue though is whether nitration affects the catalytic function and/or stability of the affected proteins. Various *in vitro* and *in vivo* studies have reported protein nitration to cause a decrease, an increase, or exert no effect on catalytic activity [15–18]. In some instances, such as sarcoplasmic reticulum Ca^{2+}-ATPase and phosphorylase *b* in the rat skeletal muscle, age-related decreases in catalytic activity were initially attributed to an increase in tyrosine nitration [4,19], however, subsequent studies suggested that oxidation of certain other amino acid residues rather than nitration of tyrosine was responsible for the decreased activity [20, 21]. Nitrohydroxylation of SCOT tryptophan 372 in the rat heart was found to be associated with an elevation rather than a decline in SCOT catalytic activity [14].

In this context, the present study was undertaken to address the following related issues: (i) whether SCOT nitration in tissues other than the heart also occurs at the tryptophan residues; (ii) whether the amount of SCOT nitration varies during the aging process and whether food restriction, which is known to extend the life span of rats [22], affects the level of such nitration; and (iii) whether SCOT catalytic activity and stability are affected by nitration and/or age of the animals.

Materials and Methods

Reagents

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich Co (St. Louis, MO). Suppliers of other materials were: acrylamide/Bis solution 40% T, 3.3% C, and broad range of prestained molecular weight markers (myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme and aprotinin, with molecular masses of 209, 124, 80, 49.1, 34.8, 28.9, 20.6 and 7.1 kDa, respectively), Bio-Rad (Hercules, CA); Immobilon PVDF transfer membranes (0.45 μm), Millipore Corp. (Billerica, MA); BioLight films, Kodak (Eastman Kodak, Rochester, NY); mouse monoclonal anti-3-nitrotyrosine, clone 1A6, Upstate (Lake Placid, NY); goat polyclonal anti-mitochondrial creatine kinase, Santa Cruz Biotechnology (Santa Cruz, CA); anti-horseradish peroxidase conjugated, goat anti-rabbit and anti-mouse IgG (H+L), Pierce (Rockford, IL); ECL Plus, Amersham Biosciences (UK); Percoll and chromatofocussing reagents, Amersham Corp. (Arlington Heights, IL); sequencing grade modified trypsin, Promega (Madison, WI); pronase from *Streptomyces griseus* and complete protease inhibitor cocktail, Boehringer Mannheim (Indianapolis, IN); 5-nitrotryptophan, WAKO Pure Chemical Industries (Richmond, VA). Rabbit polyclonal anti-SCOT antibody was produced against the SCOT synthetic peptide, KGPRFEKRIERLTTRDSP, conjugated to keyhole lymph hemocyanin, KLH, BioSource International (Camarillo, CA). The IgG fraction from rabbit immune serum was purified by ammonium sulfate precipitation and ion-exchange chromatography [23]; antibody was stored in 50% (w/v) glycerin at −80°C. N-terminal sequencing of proteins electroblotted onto PVDF membrane was performed at the Microchemical Core Facility Laboratory of the University of Southern California.

Animals and tissues

Male rats (Fischer 344) aged approximately 4-, 13-, 19- and 24- months were obtained from the National Institute on Aging-National Institutes of Health and housed at the animal facility of the University. For large-scale purification of SCOT, 200 rat kidneys were purchased from Pel-Freez Biologicals (Rogers, AK), and shipped overnight in ice-cold antioxidant buffer (50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA and 0.1 mM butylated hydroxytoluene) and used for the mitochondrial isolation shortly after delivery.

Isolation of mitochondria and preparation of soluble proteins

For each preparation, kidneys were pooled from two animals and placed in ice-cold antioxidant buffer, containing 150 mM potassium phosphate, 2 mM EDTA, and 0.1 mM butylated hydroxytoluene, pH 7.4. Kidneys were homogenized in isolation buffer consisting of 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, 10 mM EGTA, 0.5 mg/ml bovine serum albumin, pH 7.4. To isolate mitochondria, homogenates were centrifuged at 600 g for 10 min and the resulting supernatants at 8500 g for 10 min. Mitochondrial isolation was completed within 1 h after removal from the animal. Mitochondrial pellets were resuspended in homogenization buffer at concentrations of 5–10 mg/ml protein, and stored in small aliquots at −80°C. To isolate soluble proteins, mitochondria were sonicated twice (duty cycle 30, output control 5) in ice for 30 sec, and centrifuged at 100,000 g for 1 h at 4°C to separate the soluble proteins from the pelleted, membrane-bound proteins. Supernatants were collected and the pellets were resuspended in a buffer consisting of 50 mM imidazole (pH 7), 50 mM sodium chloride and 5 mM 6-aminohexanoic acid, followed by sonication and ultracentrifugation, as described above. Supernatants from both ultracentrifugations were combined, and the protein concentration was measured immediately. Samples were stored at −80°C until used.

Purification of SCOT protein from kidney mitochondria

Purification of SCOT was achieved by a procedure similar to that used previously for the rat heart [14]. Mitochondrial soluble proteins (~300 mg) were dialyzed against 25 mM imidazol buffer, containing 0.2 mM phenylmethanesulphonylfluoride (PMSF), pH 7.85, for 2 h, and applied onto a chromatofocusing column (10×250 mm), equilibrated with the same buffer, and eluted with 400 ml of Polybuffer 74 (dilution 1:10), pH 3.9 at a flow rate of 25 ml/h, and 4 ml fractions were collected. Measurements of pH were made in every fifth fraction. Each consecutive fraction was subjected to one-dimensional SDS-PAGE electrophoresis. Gels were electrotransferred onto a PVDF membrane for immunodetection with the anti-SCOT antibody. The fractions containing the SCOT protein were then pooled, and concentrated in a volume of 200 µl, using Centricon 30 concentrators. Gel filtration, i.e. separation of proteins according to their size, was performed at room temperature using a Shimadzu Class VP HPLC system and BioSep-SEC-S 3000 gel permeation column (5µm, 7.5×300 mm) obtained from Phenomenex (Torrance, CA). The column was equilibrated with 25 mM Tris buffer, pH 7.4, containing 75 mM NaCl, at a flow rate of 0.5 ml/min. Fifty µl of concentrated samples, containing the SCOT protein bands were injected onto the column, and the absorbance (200–600 nm) was monitored with a diode-array UV detector. Fractions (0.5 ml) of the eluate were collected from the gel filtration column in between 12 and 20 min. Several consecutive injections of 50µl were made and fractions containing most pure SCOT were pooled, aliquoted and stored at −80°C. The purity of SCOT was assessed by SDS-PAGE. This procedure yielded ~300 µg of electrophoretically pure (>75%) SCOT protein.

SCOT autolytic fragmentation, amino acid and MALDI-TOF analysis

To generate carboxy- and amino-terminal fragments of SCOT, the purified protein was incubated in the absence or presence of 1 mM acetoacetyl-coenzyme A for 5 min at room temperature, and then for 1 h at 70°C in 50 mM sodium phosphate buffer, pH 7.4 [24]. Proteins

were separated by one-dimensional SDS-PAGE, stained with Coomassie Blue or electrotransferred to PVDF membranes for further analysis.

The bands (10–100 μg) corresponding to the full length SCOT protein (58 kDa), the amino (37 kDa) and carboxy terminal (21 kDa) fragments were cut and placed in 0.5 ml plastic tubes containing 100 μl of 0.1 M sodium acetate buffer, pH 7.4, digested with 5% (w/w) pronase overnight at 50°C to hydrolyze the proteins into amino acids; hydrolysis was stopped by the addition of 100 μl 10% (w/v) meta-phosphoric acid. These mixtures were then centrifuged at 18,000 g for 20 min, and the supernatants transferred to autosample micro vials for injection into an HPLC apparatus fitted with a Shimadzu Class VP solvent delivery system, using a reverse phase C18 Gemini column (4.6×150 mm, 5 μm, Phenomenex, Torrance, CA). Amino acids calibration standards of tyrosine, 3-nitrotyrosine, tryptophan, 4-nitrotryptophan, 5-nitrotryptophan, 5-hydroxytryptophan, and kynurenine were prepared in 5% meta-phosphoric acid. The mobile phase for isocratic elution consisted of 25 mM monobasic sodium phosphate, 12.5% methanol, pH 2.7, adjusted with 85% phosphoric acid with a flow rate of 1 ml/min. Under such conditions the separation was completed in 30 min, 5-nitrotryptophan being the last eluted peak, with a retention time of approximately 27 min. Amino acids were detected with a model 5600 CoulArray electrochemical detector (ESA, Chelmsford, MA), equipped with a four-channel analytical cell, using potentials of +600, +700, +800 and +900 mV. With the signal to noise ratio of 4:1, the lower limit for electrochemical detection was 250 fmol for 3-nitrotyrosine, 4- and 5-nitrotryptophan, and 200 fmol for tyrosine, tryptophan, 5-hydroxytryptophan and kynurenine. Each sample was injected twice and the peak areas were averaged.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were obtained and analyzed as described in [14] and [25].

Electrospray Ionization tandem mass spectrometry (ESI-MS/MS)

In-gel tryptic digestion of purified full length, carboxy-terminal and amino-terminal fragments of SCOT, analysis of tryptic peptide sequence tags by tandem mass spectrometry, and protein identification were performed, as described previously [14,26]. Briefly, proteins from Coomassie stained gel pieces were reductively alkylated and digested with sequencing grade trypsin overnight at 37°C. Tryptic digest products were extracted, dried and resuspended in 10 μl of 0.1% (w/v) formic acid. Chromatographic separation of the tryptic peptides was achieved using a ThermoFinnigan Surveyor MS-pump in conjunction with a BioBasic-18 reverse phase capillary column (100×0.18 mm, ThermoFinnigan). Mass analysis was performed using a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer equipped with a nanospray ion source employing a 4.5 cm needle, using data-dependent acquisition mode.

Protein identification was carried out with the MS/MS search software Mascot (Matrix Science), with confirmatory or complementary analyses by TurboSEQUEST (Bioworks Browser 3.2, build 41, from ThermoFinnigan). Alkylated modification (carbamidomethyl) was designated as fixed; in contrast, oxidations of methionine and tryptophan as well as nitration of tyrosine and tryptophan were regarded as variable modifications in the TurboSEQUEST search. Rat protein database, complemented with the non-redundant protein database were received from the NCBI server. The theoretical *m/z* values for the peptides and their fragmentation ions were assessed using “MS/MS Fragment Ion Calculator” from The Institute for Systems Biology at http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html. Acceptable cross-correlation scores Xcorr using TurboSEQUEST for the positive identification of a modification were set at >1 and > 2 for a singly and doubly charged ion, respectively. In addition, MS/MS spectra of particular interest were inspected manually.

Western Blot analysis

One dimensional SDS-PAGE was performed according to the method developed by Schägger and Jagow [27]. Proteins were separated on 1.0-mm gels, consisting of 4% stacking and 10% separating gels, using a Bio-Rad Miniprotean III gel apparatus. For each experiment, one gel was stained with Coomassie Blue, and the other one was electrotransferred to a PVDF membrane to perform immunodetection steps. Protein electrotransfer onto PVDF membranes was performed at 4°C in a buffer containing 16 mM Tris base, 120 mM glycine and 10% (v/v) methanol at a constant current of 120 V for 80 min. For immunodetection, PVDF membranes were incubated with blocking solution containing 5% dry milk and 0.1% Tween 20 in Tris Buffered Saline (TBS, 10 mM Tris/HCl buffer, pH 7.5, and 150 mM NaCl) for 45 min at 37°C, or overnight at 4°C. Membranes were then quickly rinsed in distilled water and incubated with the primary antibody (anti-3NT-1:2,000 dilution, and incubation at 37°C for 1 h, or overnight at 4°C; anti-SCOT serum-1:1,000 dilution, and incubation at 37°C for 1 h). After quickly rinsing in distilled water, and washing 4–5 times, 5 min each, with Tween-TBS, PVDF membranes were incubated with the appropriate secondary antibody (HRP-conjugated goat anti-mouse IgG (H+L)- dilution of 1:20,000 and incubation for 1 h at 37°C; HRP-conjugated goat anti-rabbit-dilution of 1:100,000 or 200,000 and incubation for 1 h at 37°C). After vigorous washing with Tween-TBS for 5 min 4–5 times, the membranes were developed using the chemiluminescence detection kit, ECL-Plus. Images of the immunoblots were digitized by a flatbed scanner (Epson 2450). Analysis of densitometric data was performed using the software LabWorks 4.0.0.8. Amounts of SCOT protein and nitration in different samples of mitochondrial matrix were calculated by comparisons with standard curves fitting increasing amounts of purified SCOT (10 to 40 ng) or nitrated BSA (30 to 70 ng) and mitochondrial matrix extracts from 4-month-old rat (2.5 to 15 μg). The relation between amounts of protein and band densities was found to be exponential, rather than linear, within a limited dynamic range of amounts of protein (indicated above) and was also dependent on the duration of film exposure during the chemiluminescence reaction.

The specificity of the anti-3NT antibody was established by following controls: immunostaining was abolished in the absence of the primary antibody; after pre-incubation of the primary antibody with free nitro-tyrosine; and after reduction of the membrane with dithionite [14]. In addition, the positive (experimentally nitrated BSA) and negative (non-nitrated BSA) controls were used routinely in Western blot analysis.

Measurement of SCOT activity

SCOT catalytic activity was measured by following the appearance of the magnesium-acetoacetyl-CoA complex at 313 nm at 30°C, according to the procedure of Williamson [28]. Briefly, the final concentration of reactants in the assay mixture (0.5 ml) was: 100 mM Tris-HCl pH 8.5, 10 mM magnesium chloride, 4 mM iodoacetamide, 0.2 mM succinyl-Coenzyme A, and 50 mM acetoacetate. Various protein concentrations were tested to obtain a linear reaction for 2 min; 40 μg of soluble mitochondrial proteins from kidney were found to be the optimal amount to achieve linearity of the reaction. After initiation of the reaction by acetoacetate and succinyl-CoA, the change in absorbance was followed at 313 nm for 2 min using a Beckman DU-640 spectrophotometer. Enzyme assays were carried out in duplicate and the average absorbance was used for calculations. SCOT catalytic activity refers to the activity expressed as nmol acetoacetyl-CoA formed per min per mg mitochondrial matrix protein. The specific activity of SCOT was expressed as nmol acetoacetyl-CoA formed per min per mol of enzyme (min^{-1}). The molar content of SCOT in samples of the soluble fractions of rat kidney mitochondria was estimated in Western blots using the anti-SCOT antibody, and purified SCOT as the standard, and expressed as pmol SCOT per mg mitochondrial soluble proteins.

Results

Western blot analysis of protein nitration in kidney mitochondria from young and old rats

Equal amounts of mitochondrial soluble proteins (15 μg) from the young (4-month-old) and aged (24-month-old) rats were separated by SDS-PAGE and processed for Western blot analysis, using an anti-3NT antibody. A single major band with a molecular weight of ~ 58 kDa was found to be highly immunoreactive (Fig. 1). In addition, several faint immuno-positive bands with molecular weights of ~20–30 kDa could be detected when the Western blot analyses were performed with protein amounts >25 μg and/or the duration of chemiluminiscent reaction was prolonged (data not shown). The specificity of the anti-3NT antibody was ascertained by a series of positive and negative controls, as described in Materials and Methods. Using a polyclonal anti-SCOT antibody, the anti-3NT antibody-reactive 58 kDa band was identified to contain SCOT protein (Fig. 1). Quantification of immunodensity indicated that the amounts of nitration and SCOT protein were 55% lower in 24-month compared to 4-month-old rats.

Characterization of SCOT protein

To identify the nitrated amino acid residue(s) and to locate the site of nitration within the peptide, SCOT protein was purified from mitochondria of 200 rat kidneys, as described in Materials and Methods. The separation of soluble proteins from the kidney mitochondria by column chromatofocussing confirmed our original finding that SCOT was the major protein reacting with 3NT antibody (Fig. 2). In addition, a second nitrated protein with molecular weight of 29 kDa was identified by N-terminal sequence analysis to be enoyl-CoA hydratase, however, due to its low abundance further characterization (e. g., identification of nitration site) was abandoned. Purified SCOT from rat kidney mitochondria was submitted to a set of procedures (autolytic fragmentation, amino acid and MALDI-TOF analysis), used previously for the characterization of SCOT in rat heart mitochondria [14]: the results are presented in Table 1. Purified SCOT was found to be a dimer (MW 120 kDa) with a subunit MW of 58 kDa and an isoelectric point (pI) of 7.0. Based on MW or pI, the nitrated SCOT was indistinguishable from the non-modified form.

SCOT protein was susceptible to autolytic fragmentation during incubation at 70°C with acetoacetyl-Coenzyme A, producing amino- and carboxy-terminal fragments with MW of 37 and 21 kDa, respectively. Western blot analysis of fragmented SCOT showed that only the carboxy-terminal fragment exhibited positive immunoreaction with the anti-3NT antibody, suggesting that nitrated residue(s) was located exclusively in this fragment. Amino acid analysis of purified SCOT, full length as well as amino and carboxy terminal fragments, was performed by HPLC-electrochemical detection. Calibration standards were: tyrosine, 5-hydroxytryptophan, 3NT, tryptophan, synthetic nitrohydroxytryptophan, 4- and 5-nitrotryptophan, as described in [14,25]. Amino acid analysis indicated the presence of a peak identical to the synthetic nitrohydroxytryptophan in the full length SCOT, whereas there were no peaks corresponding to 3NT, 4- and 5-nitrotryptophan, or 5-hydroxytryptophan (detection limits were ~250 fmol of analyte on column) (Fig. 3A). Identical results of amino acid analysis were obtained after enzymatic digestion of the purified carboxy-terminal fragment, generated after substrate-induced fragmentation of SCOT (data not shown). The identity of nitrohydroxytryptophan was confirmed by MALDI-TOF analysis, which indicated that the isolated nitrated amino acid residue had a mass/charge (m/z) value identical to the synthetic nitro-5-hydroxy-tryptophan. Several characteristic ions detected in the purified nitrated amino acid were: nitrohydroxytryptophan (m/z=266.25), nitroso-hydroxytryptophan (m/z=250.25), nitrene-hydroxytryptophan (m/z=234.23) and reduction product of nitrohydroxytryptophan, amino-hydroxytryptophan (m/z=236.25) generated by sodium dithionite treatment. Thus, the data on the characterization of SCOT in rat kidney (Table 1) collectively indicated that the

post-translational modification (nitrohydroxytryptophan) observed here in the kidney is similar to that reported previously in the heart [14].

Identification of SCOT tryptophan 372 as the nitrated residue

Tandem mass spectrometric analysis (MS/MS) was used to identify the site of nitrohydroxytryptophan in the SCOT polypeptide, which contains 11 tyrosine and 3 tryptophan residues. The full-length and carboxy-terminal fragment of SCOT, excised from Coomassie stained gels, were used for MS/MS analysis. Several tryptic peptides ^{366}YGDLANWMIPGK^{377} with either a +16 mass increment on methionine 373, or a 61 atomic mass unit increase on tryptophan 372 were detected. Analysis using SEQUEST did not suggest the presence of +45 atomic mass addition to tyrosine or tryptophan residues in any detectable SCOT tryptic peptides (sequence coverage was 40% and 70 % for full length and carboxy-terminal fragment of SCOT, respectively). A typical tandem mass spectrum of the peptide, exhibiting a + 61 mass increase, is shown in Fig. 3B. The most intense ion, y6 (m/z 792.1) corresponds to tryptophan carrying nitro and hydroxy groups. Although y5 or b7 ions were not detected, the possible occurrence of methionine sulfoxide (+16) and nitrotryptophan (+45) can be excluded since HPLC and MALDI amino acid analyses unambiguously demonstrated the absence of nitrotryptophan, and instead the presence of nitrohydroxytryptophan. This fragmentation pattern was similar to that exhibited by the corresponding peroxynitrite-treated synthetic peptide, which also contains nitrohydroxytryptophan [29]. Indeed, the nitro and hydroxy groups on tryptophan 372 altered the fragmentation pattern of the peptide, with y7 and y8 becoming relatively abundant, and y5 becoming virtually undetectable. Collectively, these data indicated that the tryptophan residue 372 was the site of the nitrohydroxy modification.

Effect of age and food restriction on SCOT protein amount, nitration and catalytic activity

Soluble mitochondrial proteins from the kidneys of 4-, 13-, 19- and 24-month-old rats were separated by SDS-PAGE and the gels were probed with anti-3NT, anti-SCOT or anti-mitochondrial creatine kinase (anti-mtCK) antibodies (Fig. 4A). Compared to the 4-month-old, the immunoreactivity of the 58 kDa band with anti-3NT and anti-SCOT antibody in the 19- and 24-month-old rats gradually decreased with age, while the creatine kinase reactivity remained unaltered during aging. Overall, SCOT protein amount, determined by calibration of the Western blots of purified SCOT, and normalized to the content of mitochondrial creatine kinase, declined from 36.2 pmol/mg matrix proteins in the 4- month-old to 16.3 pmol/mg in 24-month-old rats, i.e. by 55% ($p<0.001$).

Between 4 and 24 months of age, SCOT catalytic activity, measured in the soluble fraction of mitochondrial proteins, decreased from 181±8 to 99.55±6 nmol acetoacetyl-CoA/min/mg mitochondrial matrix protein, i.e. by 45% (Fig. 4B). In contrast, specific activity of SCOT (~5×10^3 min^{-1}) expressed per mol enzyme, and nitrohydroxytryptophan content (36 mmol/mol SCOT), determined in the same samples used for measuring immunoreactivity, remained unchanged as a function of age (Fig. 4C). Thus, the fraction of SCOT that is nitrated can be calculated: since the nitrohydroxytryptophan content is 36 mmol/mol SCOT and the amount of SCOT protein per mg mitochondrial matrix protein is 36.2 pmol, there is 36 pmol × 0.036 =1.3 pmol nitrated SCOT, that is 3.6% of SCOT protein is nitrated.

Comparisons between 19-month-old rats, that had been fed ad-libitum (AL) or 40% lesser amount of food since the age of 4 months, indicated that the restricted rats had 50% greater SCOT protein content than the AL-fed rats ($p<0.001$) (32.6±1.1 pmol SCOT/mg matrix proteins in FR rats versus 21.7±1.7 in the AL rats) (Fig. 5A); SCOT catalytic activity increased from 124±10 in the AL rats to 186±9 nmol acetoacetyl-CoA/min/mg mitochondrial matrix protein in the food restricted FR rats, i.e. by 50% (Fig. 5B). However the amount of SCOT

nitration and its specific activity were not affected by CR (Fig. 5C). Altogether, these data indicated that there was a striking age-related decline in the ketolytic capacity of kidney mitochondria, since SCOT catalytic activity declined by 45%. This difference seems to be attributable to the age-related decrease in the amount of SCOT protein in the AL but not in the CR rats.

Trypsin-mediated in vitro proteolysis of SCOT

Peroxynitrite-treated proteins have been reported to be relatively more susceptible to proteasome-mediated proteolysis [30,31]. Proteolytic systems involved in the degradation of oxidatively-modified proteins in the mitochondrial matrix have not as yet been well characterized, but may include the serine proteases, Lon and ClpP [32]. Nevertheless, their *in vivo* substrates remain to be clearly identified. Thus, to investigate whether nitration had an effect on stability of SCOT, we used trypsin, which, like LON and ClpP, is a serine protease. Trypsin is known to split SCOT into two relatively hydrophobic peptides, the ~32 kDa N-terminal and the ~26 kDa C-terminal [33]. Indeed, it is well documented that in addition to the autolytic site, glutamate 303, in the SCOT protein described previously [24], SCOT also contains a highly hydrophilic region with several possible proteolytic cleavage sites [34,35], in particular lysine 255, as depicted in Fig. 6.

Briefly, 250 μg of mitochondrial soluble proteins from 2-month-old rats were incubated with 5 μg of trypsin (i.e. protein:protease ratio of 50:1) in 100 mM ammonium carbonate buffer, pH 7.8, at 37°C. Aliquots of 20 μg were removed at 0, 30, 60, 90, 120, 150 and 180 min for the determination of SCOT catalytic activity. Additionally, 15 μg mitochondrial proteins were boiled in the SDS-PAGE sample buffer for separation by SDS-PAGE. As shown in Fig. 7, the total pool of full length SCOT protein, as well as the nitrated fraction, indicated by immunoblot analysis with anti-SCOT and anti-3NT antibodies, declined rapidly during the initial 90 min of incubation with trypsin, reaching virtually undetectable levels after 3 h (Fig. 7B and 7C). In the absence of trypsin, neither the amount of SCOT protein nor the catalytic activity (data not shown) were affected even after 3 h of incubation. A comparison of the rate of proteolytic degradation between SCOT and some randomly selected protein bands (labeled **a, b, c, d, e** and **f** in Fig. 7A and 7D) showed that the rate of proteolysis of SCOT was among the fastest in this group of proteins.

To investigate whether this observation was simply due to the fact that the SCOT protein itself, rather than the nitrated SCOT, is more sensitive to proteolysis, SCOT degradation was slowed down by incubating 250 μg mitochondrial proteins with 1 μg instead of 5 μg trypsin (i.e. protein:protease ratio of 250:1). Comparison of OD between the total fraction of full length SCOT (unmodified and nitrated) and the nitrated fraction showed that nitrated SCOT was more susceptible to degradation than the pooled SCOT protein (Fig. 8A, B). This inference was corroborated by the finding that the 26 kDa carboxy-terminal fragment, containing the nitrohydroxytryptophan 372, was degraded faster than the unmodified 32 kDa amino terminal fragment (Fig. 8C). Thus, nitrated SCOT seems to be relatively more susceptible to degradation than other mitochondrial proteins. The susceptibility for degradation may be linked to the presence of the nitrated residue, as indicated by the relatively faster degradation of the carboxy terminal fragment than the amino terminal fragment.

Additional experiments involved mitochondrial preparations from young or old rats as sources of proteases, the presence/absence of ATP or metal ions, and different pH. The immuno-intensity of the endogenous SCOT band was monitored by Western blotting. However, no degradation of SCOT or of other mitochondrial proteins could be detected even after incubations of up to 24 h (data not shown). A plausible explanation for the observed lack of proteolysis may be that the optimal ratios of proteolytic enzymes to their substrates were not achieved due to the relatively low concentrations of the former, and/or that degradation occurs

at extremely low rates. The identity of the specific mitochondrial protease(s) responsible for selective degradation of SCOT is presently unclear.

Discussion

The main findings of this study were: (i) SCOT, a rate-limiting enzyme in ketone body degradation, is a selective target of nitration. (ii) Although nitration was initially detected by immuno-staining with an anti-3NT antibody, subsequent amino acid, MALDI-TOF and MS/MS analysis revealed that a specific tryptophan rather than a tyrosine residue was the actual site of nitration. (iii) The amount of SCOT protein and enzyme catalytic activity (activity/mg mitochondrial proteins) decreased significantly during aging. (iv) SCOT and particularly its nitrated carboxy region was relatively more susceptible to trypsin-induced proteolysis than other randomly selected mitochondrial proteins. (v) The age-related losses in the amount of SCOT protein and catalytic activity were prevented by reduction in food intake.

Nitration of SCOT was initially detected in the heart and kidney of streptozotocin and endotoxin-treated rats by staining with an anti-3NT antibody [36,37]. In both studies, SCOT nitration was reported to be relatively higher and SCOT activity to be lower in inflammatory or diabetic conditions. Decreased activity was ascribed to nitration because the amount of SCOT protein was not affected. Although in the current study, nitration of SCOT was also initially detected by staining with anti-3NT antibody, the presence of 3NT could not be confirmed by subsequent biochemical analysis. HPLC amino acid analysis and mass spectrometric studies of the purified SCOT carboxy region indicated that a specific tryptophan residue (372), among a total of 11 tyrosine and 3 tryptophan residues present in the SCOT protein, was the sole residue exhibiting nitration. Although the nature of the mechanism underlying this specificity is unclear, it is possible that an intramolecular electron transfer from the adjacent methionine 373 may have facilitated the nitration of tryptophan 372, by a process similar to that reported for the nitration of a tyrosine residue adjacent to a methionine residue [38]. A practical implication of our finding is that positive reactivity with anti-3NT antibodies by itself is an insufficient basis to conclude that a tyrosine residue is indeed the site of a nitro addition. Such cross-reactivity may be due to a structural similarity between the benzyl rings of tryptophan and tyrosine carrying nitro- and hydroxy-groups [14]. Whether the nitrohydroxy modification of tryptophan is a ubiquitous phenomenon, occurring in some other proteins, that have previously been reported to be nitrated on the basis of reactivity with anti-3NT antibody [1,3,5,7–9], remains a possibility.

It should also be noted that, in addition to the potential pitfalls associated with the use of the anti-3NT antibody, tandem mass spectrometry might also yield false positive identification of nitrated proteins [39–41]. Accordingly, the present and our previous study used a combination of different biochemical approaches to ascertain the identity and actual occurrence of nitrohydroxytryptophan: (i) HPLC amino acid analysis of the purified SCOT carboxy region allowed the isolation of the nitrated residue, and subsequent MALDI analysis of the HPLC fraction of interest helped in determining the nature of the modifications in the residue. (ii) Furthermore, a peptide, corresponding to the nitrohydroxytryptophan-containing peptide identified *in vivo*, was synthesized and nitrated with peroxynitrite, thereby allowing direct comparison between fragmentation patterns of the *in vivo* and *in vitro* modified peptides, and establishing the occurrence of a novel posttranslational modification [14,25,29]. It should be noted that the exposure of synthetic SCOT peptide to peroxynitrite resulted in ubiquitous oxidation of methionine residues [29]. The nitrohydroxytryptophan-containing-peptide also showed the presence of methionine sulfoxide or sulfone groups. In contrast, MS/MS data on purified SCOT, from the rat kidney (present work) and heart mitochondria [14], clearly indicated the absence of simultaneous modifications, i.e., nitrohydroxytryptophan plus methionine sulfoxide. Although peptides, containing methionine sulfoxide only, were detected,

their abundance was far lesser than that present in the synthetic peptide treated with peroxynitrite. Also taking into account that the abundance of nitrohydroxytryptophan-containing peptides is quite low, the likeliness of detecting peptides with both modifications would seem to be quite weak. One possibility is that methionine oxidation was introduced by extraneous factors during tissue handling.

It is widely supposed that ROS/RNS-induced post-translational structural modifications in proteins result in enhanced proteolysis and a consequent loss of catalytic function. Thus, the observed, relatively large age-related decrease (55%) in the amount of SCOT protein raises the question whether the presence of nitro and hydroxy groups on the tryptophan residue might act as a marker for selective proteolysis. The steady-state amounts of post-translationally modified proteins are known to be dependent on a combination of factors, such as the rates of synthesis of mature non-modified protein, oxidation/nitration and degradation/turnover. The full-length SCOT was found here to be relatively more susceptible to trypsin-mediated proteolysis *in vitro* than the other randomly selected kidney mitochondrial proteins. The carboxy-terminal fragment of SCOT, containing the nitrohydroxytryptophan 372, was also more susceptible to proteolysis than the non-nitrated amino-terminal fragment. According to the PeptideCutter algorithm (http://us.expasy.org/tools/peptidecutter/), SCOT peptide contains 24 potential trypsin cleavage sites in the amino- terminal fragment compared to 14 in the carboxy-terminal fragment (100% probability). Thus, it seems implausible that the preferential degradation of the nitrated fragment of SCOT, compared to the non-nitrated fragment, may be due to the presence of relatively more cleavage sites in the carboxy-terminal fragment. Nevertheless, the molar content of nitrated SCOT *in vivo* remained unaltered during aging, thereby suggesting that non-nitrated and nitrated SCOT protein are equally susceptible to proteolysis. Thus, even though *in vitro* data suggest that nitrated SCOT is more susceptible to degradation, the functional consequences of SCOT nitration *in vivo* remain presently unclear. A partial association between SCOT nitration and the age-related decline in SCOT protein remains plausible, as low molecular weights SCOT peptides might remain undetected in the immunoblots.

Additional factors that may contribute to the observed age-related decline in SCOT protein include (i) an age-related increase in acyl-CoA induced SCOT fragmentation (autolysis), (ii) an age-associated decline in gene transcription, or (iii) a loss in renal mass/mitochondria. For SCOT autolysis to occur under physiological conditions, concentration of the first substrate, succinyl-CoA, should exceed the concentration of acetoacetate, the second substrate [24]. Nevertheless, in the absence of reliable *in vivo* information about the relative concentrations of the substrates in the aged animals, the potential contribution of autolysis remains speculative. The notion, that an age-related decline in gene transcription in the kidney may be a contributory factor in the loss of SCOT protein, currently lacks support because quantitative analysis of transcripts in the kidney of young and aged rats did not provide any supportive evidence for a corresponding decrease in the abundance of SCOT transcripts [42]. Furthermore, despite the aging-associated loss of nephrons and mitochondria in the kidneys of rodents [43], many genes remain stably expressed or are up-regulated and only a few undergo down-regulation [42,44]. Thus, overall, factors involved in the decline in SCOT protein content during aging *in vivo* remain to be identified.

The age-associated alterations in SCOT protein and activity may contribute to the well-documented functional impairments described in the kidney of aged Fischer rats [45]. Indeed, diminution in SCOT catalytic activity may lead to a rise in levels of ketone bodies within the kidney of older animals, and since they are strong acidic compounds, the intra-mitochondrial pH may decrease, thereby adversely affecting the function of some enzymes. Calorie restriction (CR), a regimen that extends life span of rats and mice [22], is also known to preserve the renal function of Fischer rats [45]. In this context, restoration of SCOT protein levels and catalytic

activity in the kidney mitochondria from CR rats to the levels of young rats correlates with the functional improvements in this organ after long-term food restriction.

Another notable finding of this study was that the age-related changes in the amounts of SCOT protein, nitration content and catalytic activity in the kidney differ from those observed previously in the heart [14]. For instance, the amount of SCOT protein in the heart was found to remain unchanged with age while the molar content of nitrohydroxytryptophan 372 and SCOT specific activity increased significantly. The specific activity of nitrated SCOT, normalized as mol/mol SCOT protein was ~ 12 times higher than that of the non-nitrated SCOT. In contrast, in the kidney, the amount of SCOT protein decreased with age, whereas molar content of nitrated SCOT and its specific activity remained unaffected. Therefore, the functional impact of nitration itself cannot be established to the same extent in the kidney, as was demonstrated previously in the heart mitochondria, due to this lack of changes. An additional difference between the two organs was that CR had no effect on SCOT protein amount in the heart, whereas CR prevented the age-related loss in SCOT protein levels in the kidney. Such apparent contrast in the two organs is not surprising since (i) mitochondria among different tissues are well documented to be functionally heterogeneous [46,47], and (ii) heart and kidney differ in their energy requirements and patterns of aging. Thus, our results suggest that the mechanisms governing the degree of SCOT nitration are tighter in kidney mitochondria than in heart mitochondria. The regulation of SCOT protein amounts also seemingly differ in these two organs. Yet, the protein undergoes the same type of age-related structural alteration, namely a nitrohydroxy modification of tryptophan 372 in the kidney as well as in the heart.

To conclude, the present study confirms the presence of a novel post-translational modification, namely nitro-hydroxylation of a single tryptophan residue, in the enzyme, SCOT, in the rat kidney mitochondria. The amount of SCOT protein decreased dramatically during aging, which would likely attenuate the capacity of the kidney to utilize ketone bodies. Notably, this age-related decline was prevented by a reduction in food intake.

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List of Abbreviations

SCOT	succinyl-CoA:3-ketoacid coenzyme A transferase
3NT	3-nitrotyrosine
AL	ad libitum
CR	caloric restriction
FR	food restriction
MS/MS	tandem mass spectrometry
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight.

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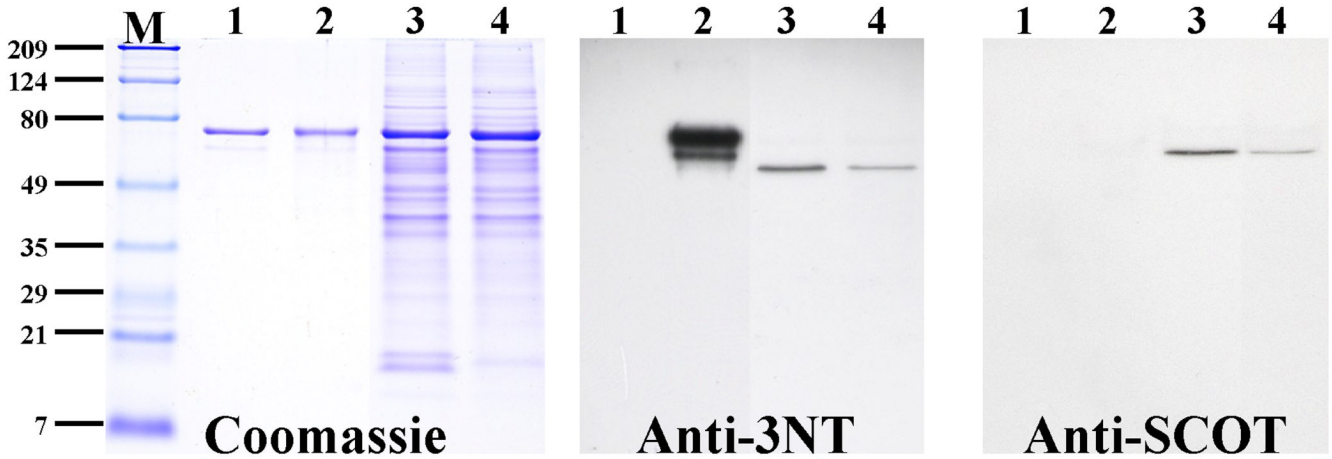

Figure 1. Western blot analysis of nitrated proteins in the soluble fraction of mitochondria from kidney of the young and old rat

Mitochondria were sonicated and centrifuged at 100,000 g. Aliquots of the resulting supernatant (soluble mitochondrial proteins), containing 15μg protein per lane, were separated by SDS-PAGE. Immunoblot analysis was performed with anti-3nitrotyrosine monoclonal antibody (**Anti-3NT**) or with polyclonal anti-SCOT antibody (**Anti-SCOT**). A Coomassie-stained gel is shown in the left panel. M refers to pre-stained molecular weight markers, myosin, β-galactosidase, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme and aprotinin, with polypeptide molecular masses of 209, 124, 80, 49, 35, 29, 21 and 7 kDa, respectively. Contents of different lanes were: 1, BSA; 2, 3NT-BSA; 3 and 4, samples from kidneys of 4- and 24-month-old rats, respectively.

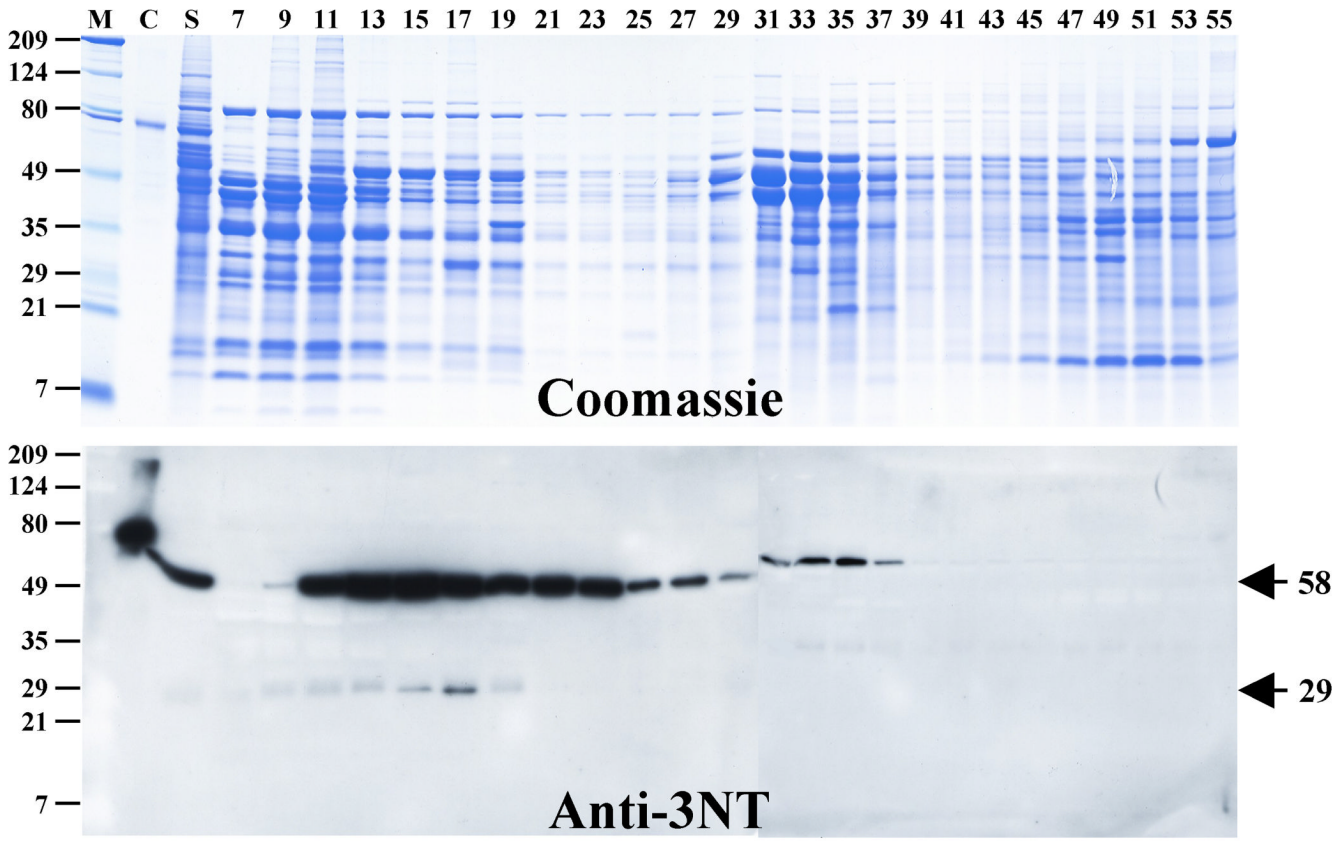

Figure 2. Purification of the 58 kDa nitrated protein from kidney mitochondria by column chromatofocussing

Soluble proteins from kidney mitochondria (300 mg) were separated by chromatofocussing, as described in the Materials and Methods section. Proteins from the collected fractions were separated by SDS-PAGE and the gels were stained with Coomassie Blue or immunostained with anti-3NT antibody. M refers to the mixture of proteins containing untreated BSA and pre-stained molecular weight markers, indicated in the legend of Fig. 1. Lane contents were: C, 3NT-BSA (0.5 μg); S, soluble proteins from kidney mitochondria (15 μg); lanes 7–55 correspond to aliquots (25 μl) of fractions from the chromatofocussing column. The arrows indicate the positions of the SCOT protein (58 kDa) and enoyl-CoA hydratase (29 kDa), which co-elutes with SCOT.

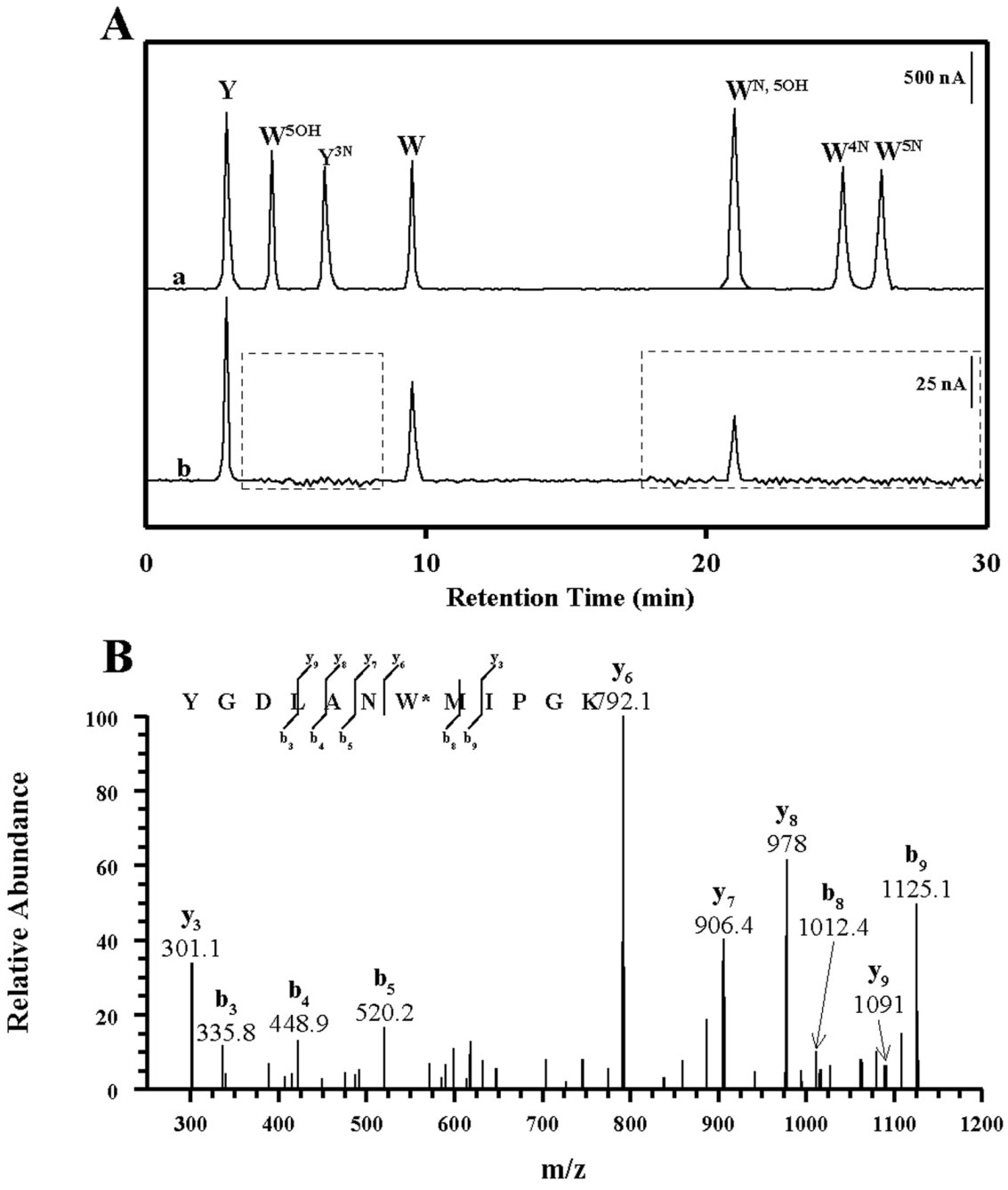

Figure 3. Presence of nitrohydroxytryptophan in SCOT protein and identification of tryptophan 372 as the site of modification

Panel **A** shows a characteristic chromatogram of amino acid analysis after enzymatic hydrolysis of the full-length SCOT protein, purified from kidney of young rats (trace b). Amino acids used as calibration standards are shown in trace a: tyrosine (Y; retention time=2.87 min), 5-hydroxytryptophan (W^{5OH}; retention time=4.5 min), 3-nitrotyrosine (Y^{3N}; retention time=6.37 min), tryptophan (W; retention time=9.5 min), 5-hydroxy-nitrotryptophan ($W^{N,5OH}$; retention time=21 min), and 4 or 5 nitrotryptophan (W^{4N}, W^{5N}; retention times= 24.87 and 26.25 min respectively). The vertical bars indicate the intensities from the electrochemical detector: 500 nA for the top chromatogram, and 25 nA for the bottom chromatogram, inside the dotted box.

Panel **B** shows the MS/MS spectrum for SCOT tryptic peptide Y_{366}GDLANWMIPGK$_{377}$, containing nitro-hydroxytryptophan ($[M+2H]^{2+}$ ion at *m/z* 713.3 and X_{corr} is 2.31). The ion annotation is based on results presented in the SEQUEST's "display ion view" window, and corroborated by the dta file. W* indicates a nitro-hydroxytryptophan.

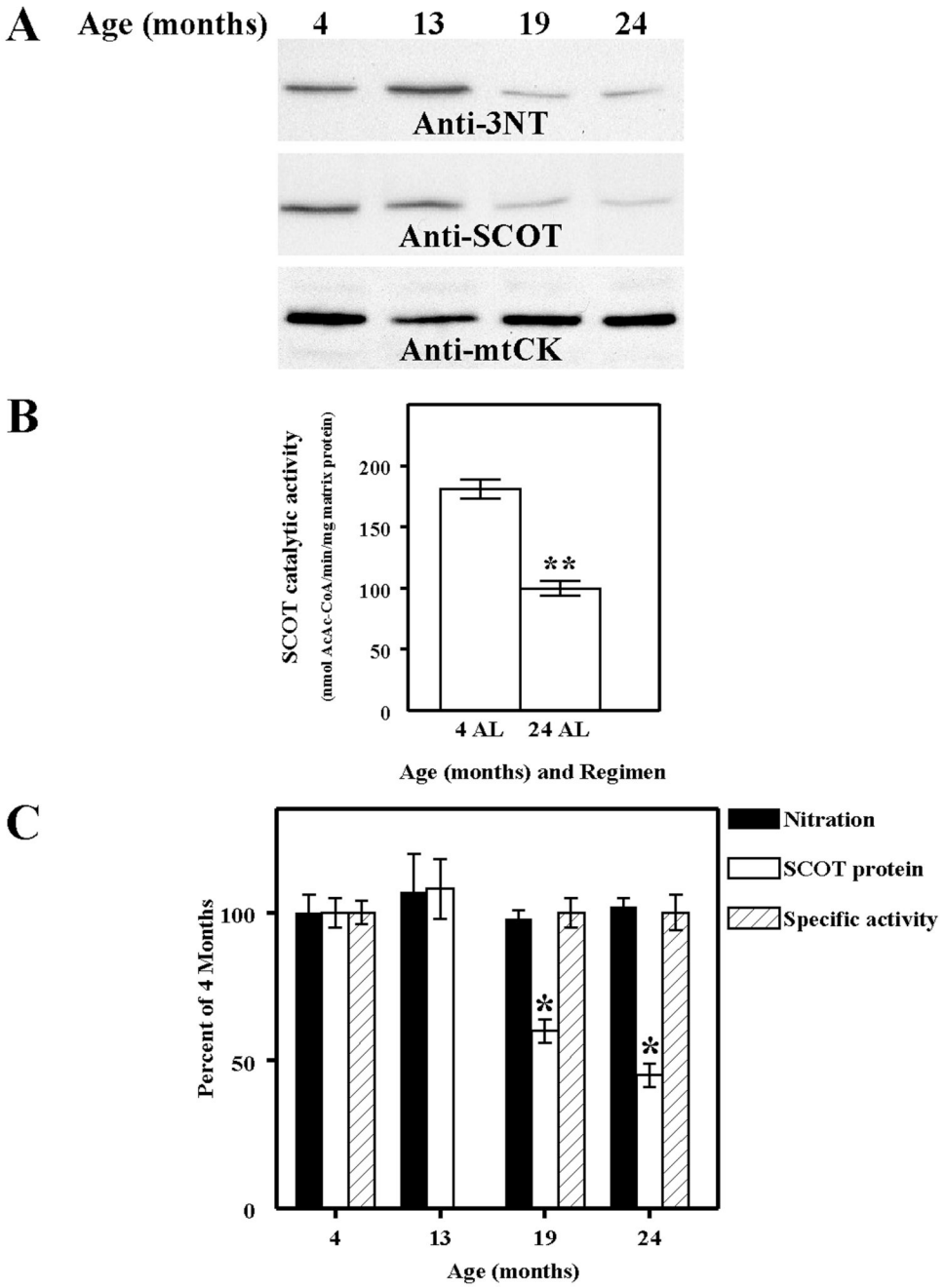

Figure 4. Effect of age on protein amounts, nitration, catalytic and specific activity of SCOT in kidney mitochondria

Panel **A**: Immunoblot analysis of kidney mitochondrial proteins from rats of various ages, demonstrating decreased amounts of anti-3NT and anti-SCOT reactivity at older ages, and stable levels of anti-mitochondrial creatine kinase (anti-mtCK) reactivity during aging. Panel **B**: Catalytic activity of SCOT (determined in samples of soluble proteins from kidney mitochondria, and expressed as per mg matrix proteins), declined by 45% in ad libitum-fed (AL) rats between 4 and 24 months of age (Values represent mean ± standard deviation of n=9 for activity assays; ** $P<0.001$). Panel **C**: Nitration content, protein amounts, and specific activity of SCOT are presented as percentages of the amounts present in the 4-month-old rats

($5 \pm 0.2 \times 10^3$ min^{-1}, 36 ± 2.5 mmol/mol SCOT and 36.2 ± 1.9 pmol/mg matrix proteins, respectively). All values represent mean ± standard deviation of n=6 for immunoblot analysis and n=9 for activity assays; * indicates significant difference vs. 4-month control (P<0.001). Activity in 13-month-old rats was not determined.

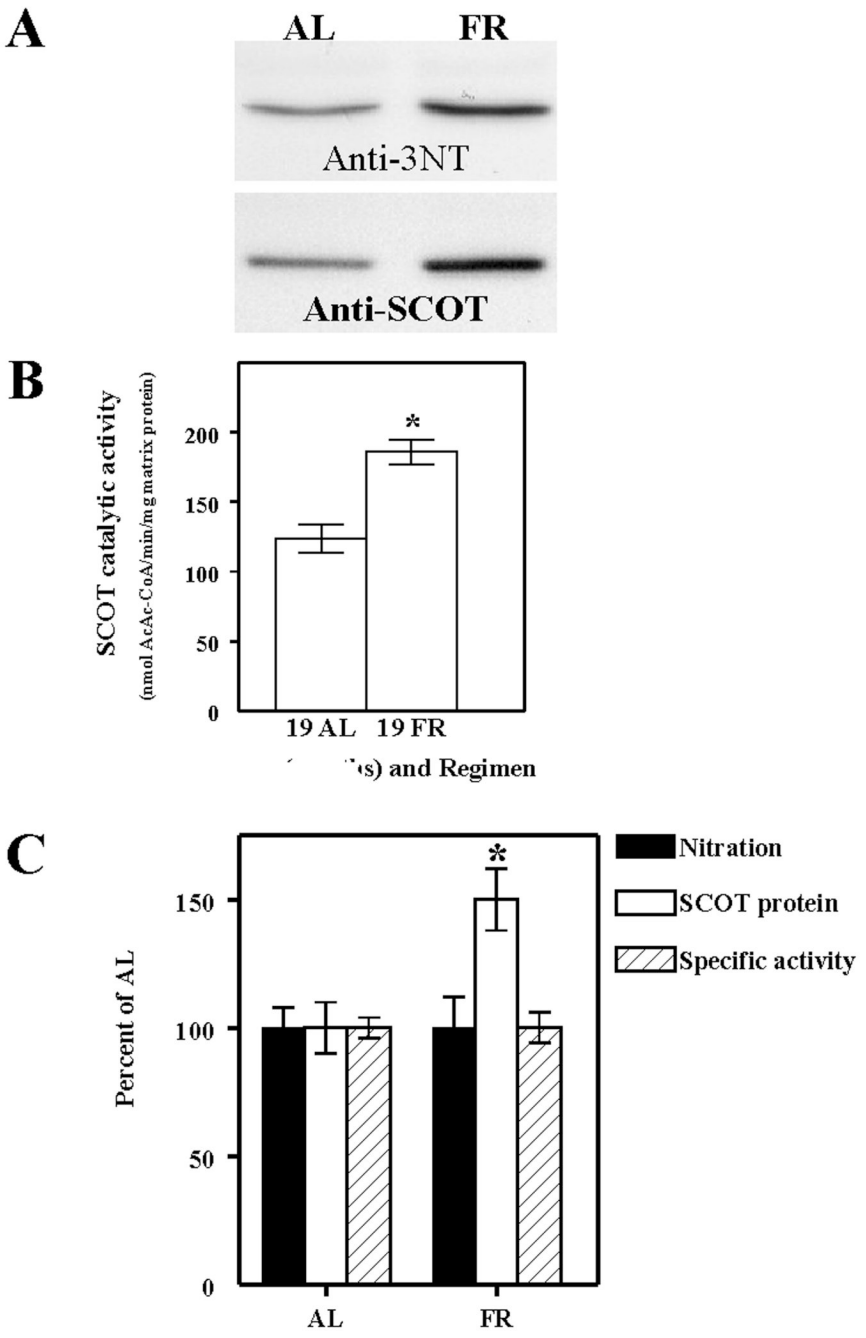

Figure 5. Effects of food restriction on protein amounts, nitration, catalytic and specific activity of SCOT in kidney mitochondria

Soluble proteins from kidney mitochondria, isolated from 19-month- old, ad libitum-fed (AL) and food restricted (FR) rats, were compared for the density of the immunoblots prepared with anti-3NT and anti-SCOT antibodies (Panel **A**) and catalytic activity of SCOT (Panel **B**). In the food restricted (FR) rats, at 19 months of age, SCOT protein levels and catalytic activity increased by 50% in comparison to the AL-fed rats. Percent differences between AL and FR groups in amounts of nitration, SCOT protein, and specific activity are presented in the graph (Panel **C**). In the AL-fed rats, specific activity of SCOT was $5.2 \pm 0.5 \times 10^3$ min^{-1}, the nitration content was 36 ± 3 mmol/mol, and the amount of SCOT protein was 21.72 ± 1.5 pmol/mg.

Values represent mean ± standard deviation of n=6 for immunoblots and n=9 for activity assays; * indicates significant difference vs. AL ($P<0.01$).

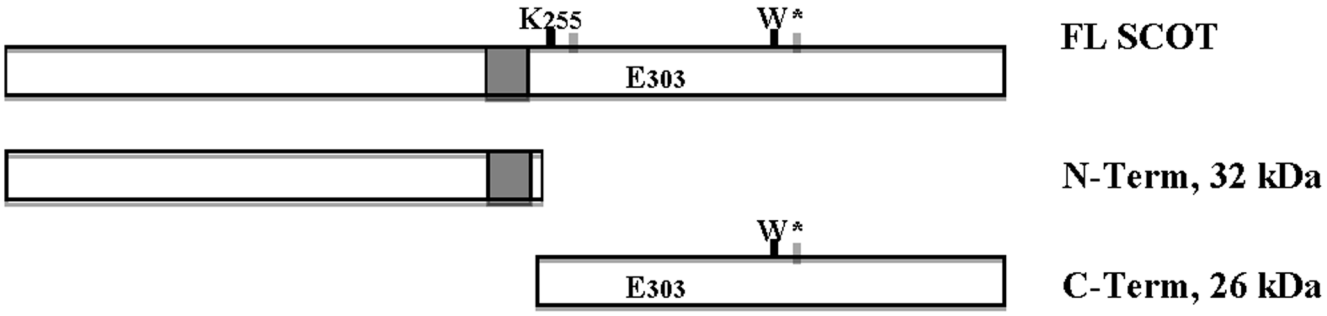

Figure 6. Scheme of full length SCOT and the two hydrophobic peptides generated upon proteolysis

Full length (FL) SCOT contains a highly hydrophilic region, which is susceptible to proteolysis. The main cleavage site is lysine 255 (K255), and upon degradation, two relatively hydrophobic peptides are generated: the N-terminal (32 kDa) and the C-terminal fragments (26 kDa). The N-terminal fragment contains the peptide recognized by the anti-SCOT antibody (represented as a grey box). The C-terminal fragment contains nitrohydroxytryptophan 372 (W*), recognized by the anti-3NT antibody, and the autolytic site, glutamate 303 (**E**).

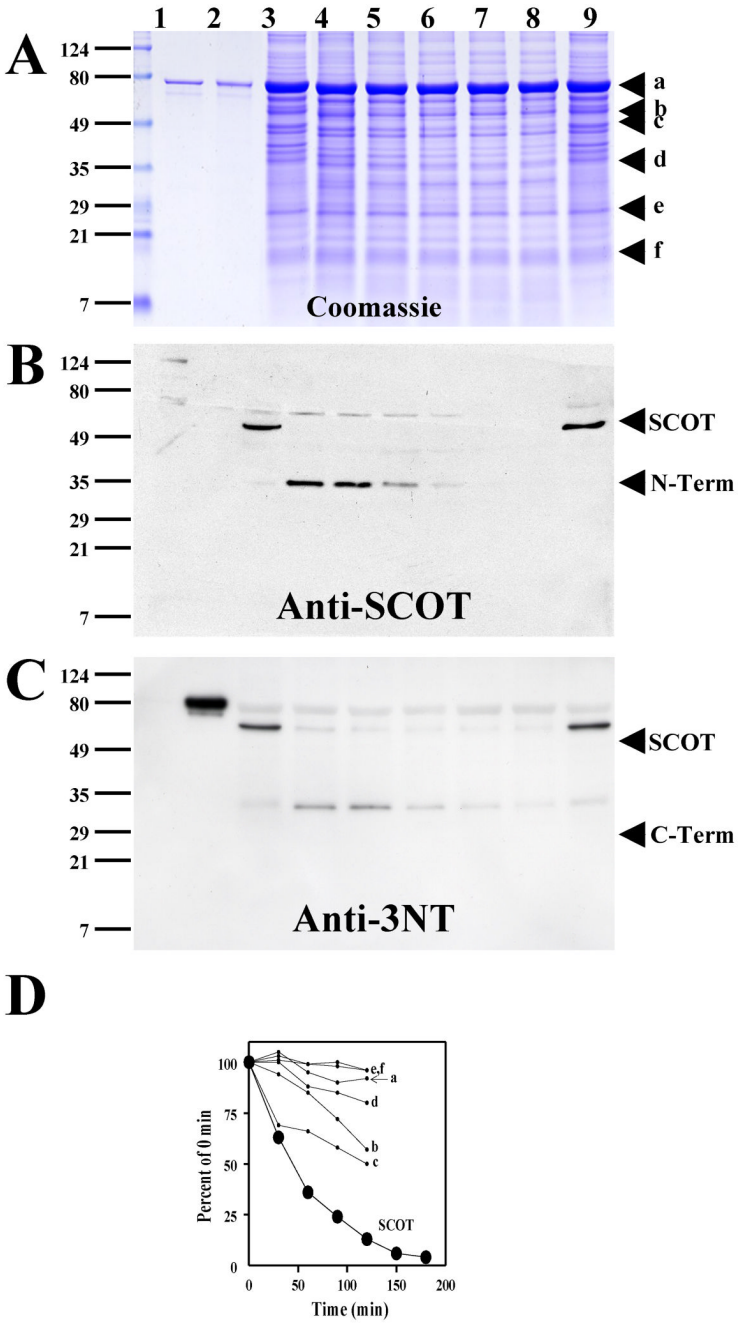

Figure 7. Degradation of SCOT by limited proteolysis *in vitro*

Soluble mitochondrial proteins from kidney mitochondria were incubated with trypsin at a protein:protease ratio of 50:1 for 180 min. Samples were then subjected to SDS-PAGE and stained with Coomassie Blue (**A**), or analyzed by immunoblotting for SCOT protein levels (**B**) and 3NT content (**C**). Non-nitrated and nitrated BSA in lanes 1–2 served as negative and positive controls, respectively; lanes 3 and 9 contain non-treated soluble proteins at 0 and 180 min. Samples taken at 0, 30, 60, 90 and 120 min during incubation with trypsin are in lanes 3–8, respectively. Randomly selected protein bands **a–f**, indicated by arrows on the right of the Coomassie stained gels (**A**) were quantitated by densitometry. SCOT full length (SCOT) and carboxy-terminal domain (C-Term, ~26 kDa) are indicated on the immunoblot (**B**) by the

arrows on the right. Panel **C** shows a comparison of time-dependent degradation of SCOT (SCOT activity and protein, large circles) with that a set of selected proteins, **a, b, c, d, e** and **f** (small circles), values represent percentages of the controls (0 min of trypsin incubation).

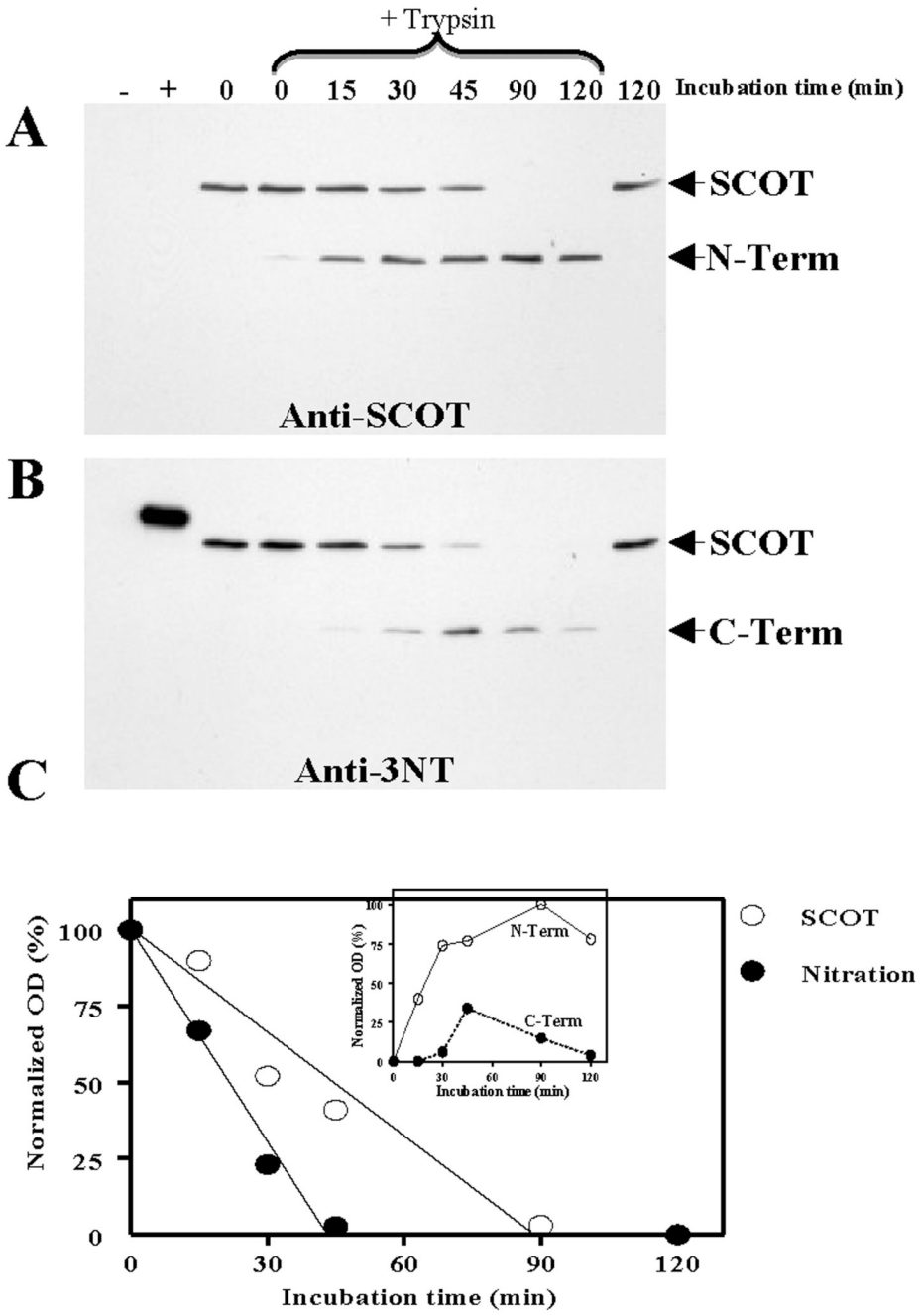

Figure 8. Degradation of carboxy and amino terminal fragments of SCOT during limited proteolysis *in vitro*

Soluble mitochondrial proteins from kidney mitochondria were incubated with trypsin at a protein:protease ratio of 250:1 for 120 min. Samples were subjected to SDS-PAGE and analyzed by immunoblotting for SCOT (**A**) and nitration (**B**) content at various time intervals. Samples were removed at 0, 15, 30, 45, 90 and 120 min after incubation with trypsin; untreated proteins were also incubated for 0 and 120 min in the absence of trypsin. BSA (−) and nitrated BSA (+) served as negative and positive controls. The positions of full-length SCOT protein (**SCOT**), carboxy-terminal (**C-Term**) and amino-terminal (**N-Term**) fragments are indicated by arrows on the right. The time-dependent disappearance of full-length SCOT band density

was normalized to the control (0 min) and presented as a percentage in panel **C**. The insert in **C** shows normalized optical densities (percent of SCOT immunointensity at 0 min) of amino-terminal (N-Term) and carboxy-terminal (C-Term) fragments of SCOT, detected by anti-3NT and anti-SCOT antibodies, respectively.

Table 1

Biochemical properties of SCOT protein purified from kidney mitochondria.

Native and Subunit MW	120 kDa and 58 kDa
Isoelectric point (pI)	7.0
N-terminal sequence	VKFYTDPVKAVEGI
Acyl-CoA-induced autolytic cleavage of SCOT	Amino-terminal 37 kDa fragment (10 tyrosine and 1 tryptophan residues) Carboxy-terminal 21 kDa fragment (1 tyrosine and 2 tryptophan residues)
HPLC-ECD amino acid analysis for nitrated and/or hydroxylated residues	5-hydroxynitrotryptophan present in full length and carboxy-terminal fragment of SCOT Nitrated derivatives of tyrosine and tryptophan are not detectable in SCOT protein
MALDI-TOF analysis of isolated nitrohydroxytryptophan from SCOT protein digest	Nitrohydroxytryptophan m/z=266.25 Nitroso-hydroxytryptophan m/z=250.25 Nitrene-hydroxytryptophan m/z=234.23 Amino-hydroxytryptophan m/z=236.25