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Nitration of Specific Tyrosine Residues of Cytochrome c Is Associated with Caspase-Cascade Inactivation

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Peroxynitrite, a potent oxidative stress inducer, inhibits the mitochondrial electron transfer, induces cell death, and is considered to be involved in the pathology of various diseases. However, the intracellular mechanisms involved in the cell death process are not fully understood. Here we demonstrate that the enhanced nitration of specific tyrosine residues of cytochrome c, which are induced by continuous peroxynitrite exposure, attenuates cytochrome c-induced caspase-9 activation *in vitro*. Interestingly, cytochrome c nitrated with a single high dose of peroxynitrite preserved its potency, while this did not occur when cytochrome c was treated with continuous peroxynitrite exposure. Although both of these experiments resulted in cytochrome c nitration at the tyrosine residues, it was found that nitration at specific residues was enhanced only when cytochrome c was exposed to continuous peroxynitrite. This is the first report to demonstrate that cytochrome c nitration affects the apoptotic pathway by means of enhancement of nitration at specific tyrosine residues. This result implies that the nitration pattern of cytochrome c may affect the efficacy of the mitochondrial pathway in apoptotic cell death.

Key words cytochrome c; nitrotyrosine; apoptosis; peroxynitrite; caspase

Peroxynitrite is a very strong oxidant, and is considered a candidate as an *in vivo* oxidant for inducing the oxidative and "nitrative" stress in various diseases, such as cardiovascular disease,[1—3] brain ischemia,[4—7] Parkinson's disease,[8—10] Alzheimer's disease,[11—14] amyotrophic lateral sclerosis,[15,16] other neurodegenerative diseases,[17,18] and sepsis.[19,20] Peroxynitrite causes nitration of free tyrosine and protein tyrosine residues. Although this reaction has been considered as the "footprint" of peroxynitrite production, it is not exclusively caused by peroxynitrite, but formed by the reaction of nitrite with hydrogen peroxide in the presence of myeloperoxidase.[21] Nonetheless, protein tyrosine nitration is a clue that reactive nitrogen species (RNS), like peroxynitrite and its equivalents, are produced and that the biological system has been damaged by RNS stresses. Some reports indicate that the nitration of protein tyrosine residues can cause some changes in function. Radi and colleagues reported that cytochrome c was nitrated at a specific tyrosine residue by a bolus peroxynitrite treatment depending on the concentration of peroxynitrite.[22] According to their results, nitration of cytochrome c resulted in loss of function as an ascorbate oxidase and in the downregulation of the oxygen consumption in mitochondrial preparation. We also independently reported that the nitration of a single tyrosine residue in cytochrome c by a relatively low dose of peroxynitrite resulted in the upregulation of its peroxidase activity for hydrogen peroxide and in the actual impairment of the membrane potential formation, which is important for ATP synthesis, in isolated mitochondrial preparations.[23]

It is well known that cytochrome c plays an important role in mitochondria-dependent apoptotic cell death. As a response to apoptotic stimuli, cytochrome c is released from the intermembrane space to the cytosol, and forms the apoptosome complex with procaspase-9 and Apaf-1 to activate caspase-9 and the downstream caspases, resulting in apoptotic death execution. One report[24] investigated the effect of cytochrome c treated with a bolus of peroxynitrite on the apoptotic cascades. In that report, cytochrome c nitrated with a bolus treatment of peroxynitrite retained its ability for caspase activation. Under the pathophysiological conditions, however, peroxynitrite production was assumed to be relatively low and sustained. The effect of low-dose and continuous exposure of cytochrome c to peroxynitrite on caspase activation was not evaluated, and the relationship between cytochrome c nitration and the caspase cascade activation has not been well investigated.

Here, we report on the nitration of cytochrome c by various methods of peroxynitrite exposure, and we analyze the relationship between tyrosine nitration and the ability of cytochrome c to cause caspase cascade activation *in vitro*. We determined that the modification pattern of cytochrome c was dependent on the duration and concentration of peroxynitrite exposure, and that cytochrome c nitration by continuous exposure to peroxynitrite attenuated its potency for caspase 9 activation. However, cytochrome c nitration by a bolus peroxynitrite treatment did not change that ability.

MATERIALS AND METHODS

Chemicals Cytochrome c (bovine heart), aprotinin, and pepstatin A were purchased from Sigma (St. Louis, MO, U.S.A.). Pronase was from Boehringer-Mannheim (Mannheim, Germany). 3-Nitro-L-tyrosine, 5-methoxytryptamine and tetranitromethane (TNM) were from Aldrich (Milwaukee, WI, U.S.A.). Staurosporine and leupeptin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). PMSF was from Nacalai Tesque (Kyoto, Japan). 3-Morpholinosydnonimine (SIN-1) was from Dojindo (Kumamoto, Japan). All other reagents were from Sigma, Bio-Rad (Hercules, CA, U.S.A.), or Amersham Biosciences

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Corp. (Piscataway, NJ, U.S.A.) All the reagents were of analytical or biochemical grade.

Peroxynitrite Preparation Peroxynitrite was synthesized as an alkaline solution based on the method of Pryor *et al.*[23,25] The solution was stored at −80 °C until use. The concentration of the peroxynitrite solution was determined spectrophotometrically by measuring the absorbance at 302 nm (ε=1670 M^{-1} cm^{-1}). Using this method, up to a 500 mM solution of peroxynitrite was obtained. The concentration of the stock solution was determined again before use, and then the stock was diluted to the desired concentration with 0.01 M NaOH on an ice bath.

Cell Culture A C6 rat glioma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in Ham's F10 medium containing penicillin and streptomycin, supplemented with horse serum and fetal bovine serum as described in the ATCC instruction. The cells were maintained at 37 °C in a humidified 5% (v/v) CO_2 incubator under a sub-confluent condition.

Preparation of Peroxynitrite- and TNM-Treated Cytochrome c Cytochrome c solution (20 μM) was prepared in PBS. For continuous treatment with peroxynitrite, 1 μl of 50 mM peroxynitrite in 0.01 M NaOH was repeatedly added to 1 ml of the cytochrome c solution (20 μM) 20 times at 30-s intervals while mixing. Because peroxynitrite is unstable and reactive at neutral pH, it is practically fully reacted or decomposed within 30 s after addition. For a continuous infusion, 20 μl of 50 mM peroxynitrite in 0.01 M NaOH was continuously infused into 1 ml of the cytochrome c solution over 20 min with a syringe pump while mixing. For a single treatment with peroxynitrite, 20 μl of 50 mM peroxynitrite was added to the cytochrome c solution all at once. For a low-dose single or control treatment, 1 μl of 50 mM peroxynitrite or no peroxynitrite was added to the cytochrome c solution containing decomposed peroxynitrite equivalent to the 19 μl or 20 μl of 50 mM peroxynitrite, respectively. After the addition of 20 μl of peroxynitrite solution, the resulting solution was confirmed to be neutral (pH 7 to 8). All the peroxynitrite-treated cytochrome c solutions were subjected to gel filtration with Sephadex G-25, or centrifugal concentration and wash with a membrane filter (polyethylenesulfonate, 5000 molecular weight cut off, Vivascience AG, Hanover, Germany). The cytochrome c concentrations were adjusted according to the absorbance at 409 nm. With the peroxynitrite treatment at the concentration range in these experiments, the maximum absorbance and wavelength of the Soret band (409 nm) showed almost no changes. The Soret band was slightly blue-shifted by less than 1 nm in wavelength. A solution of cytochrome c repeatedly treated with low-dose peroxynitrite in the presence of 5-methoxytryptamine (5MT) was also prepared in order to determine the inhibitory effect of 5MT.

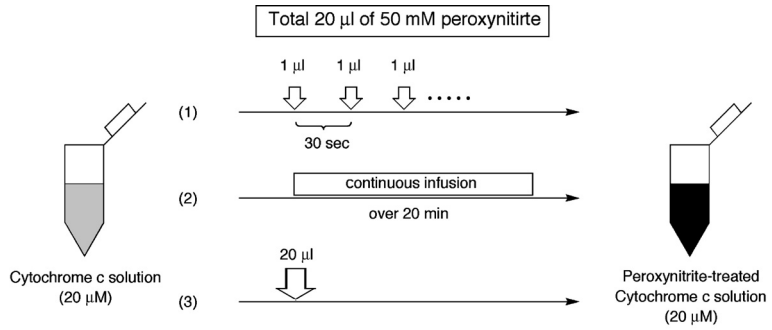
For the TNM treatment, 1 mM cytochrome c in PBS was diluted with 10 mM sodium phosphate buffer (pH 8.0) to prepare 1 ml of 20 μM cytochrome c. To this solution, 1.19 μl of 10% (v/v) TNM solution in ethanol was added, and mixed vigorously for 1 h at room temperature. The reaction was terminated by gel filtration through Sephadex G-25 with PBS, and the concentration of cytochrome c was adjusted according to the absorbance at 409 nm.

In Vitro* Caspase Activation Assay** The caspase activation assay in a cell-free system was carried out using a cytosolic fraction of C6 cells and exogenous cytochrome c.[26,27] Intact C6 cells were gently washed and harvested by scraping in PBS. The collected cells were washed with PBS again and precipitated at 200 ***g for 5 min at room temperature. The cells were then resuspended in 3 times the volume of buffer A (250 mM sucrose, 20 mM Hepes-K [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) supplemented with 4 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 0.1 mM PMSF, and 25 μg/ml *N*-acetyl-leucyl-leucyl-norleucine. The suspension was incubated on an ice bath for 15 min, and then the cells were homogenized with a glass homogenizer with 3 to 5 strokes of a pestle, or gently passed through a 22-gauge needle 15 times. The resulting cell homogenate was centrifuged at 7700 ***g*** and the supernatants were subsequently centrifuged at 100000 ***g*** for 30 min at 4 °C. The supernatant was collected as a cytosolic fraction (S-cytosol). The S-cytosol fraction did not contain cytochrome c, as confirmed by immunoblotting with the anti-cytochrome c antibody (1 : 1000 dilution, mouse IgG clone 7H8.2C12, BD Biosciences, San Jose, CA, U.S.A.). The protein concentrations of the obtained S-cytosol fractions ranged from 2.5 to 5 mg protein/ml.

For *in vitro* caspase activation assay, the peroxynitrite-treated or control cytochrome c (800 nM) was added to the S-cytosol fraction (2.5 mg protein/ml) with or without addition of 0.5 mM ATP. The mixture was incubated at 30 °C for 90 min, and the proteins were denatured by boiling for 10 min in a SDS sample buffer. The samples were subsequently subjected to SDS-PAGE (15% gel) and immunoblotting with the anti-cleaved caspase-3 antibody (1 : 1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, U.S.A.) and visualized by chemiluminescence with ECL-plus reagents (Amersham Biosciences Corp., Piscataway, NJ, U.S.A.).

Detection of Nitrotyrosine in Hydrolysate of Peroxynitrite-Treated Cytochrome c Peroxynitrite-treated cytochrome c was hydrolyzed enzymatically as reported previously.[23] The hydrolysate of peroxynitrite-treated cytochrome c was centrifuged at 175 ***g*** for 2 min, and the supernatant was analyzed by HPLC with a multi-wavelength detector (JASCO Co., Ltd., Tokyo, Japan) and an octadecylsilyl (ODS) column (TSK-GEL ODS-80Ts, 4.6×150 mm, Tosoh, Tokyo, Japan). As a mobile phase, 0.1 M potassium phosphate buffer (pH 3.5) containing 5% (v/v) methanol was used. The elution of tyrosine and nitrotyrosine was confirmed by comparison of the retention time with the authentic compounds.

HPLC and Mass Spectral Measurement of Tryptic Peptides from Nitrated Cytochrome c Peroxynitrite-treated cytochrome c (20 μM) was concentrated with a centrifugal concentrator with a polyethylenesulfonate membrane and molecular weight cut-off at 5000. The concentrated cytochrome c was washed once by a centrifugal concentrator with an ammonium carbonate solution (0.1 M, pH 8.0), and adjusted to 1.28 mM with the washing solution. A proteomic grade of trypsin (Sigma, St. Louis, MO, U.S.A.) was reconstituted with 1 mM HCl according to the manufacturer's instruction and added to the cytochrome c solution at a ratio of 1 : 100 (w/w) as the amount of proteins. The mixture was incubated at 37 °C for 16 h for tryptic digestion. The obtained tryptic peptides were analyzed with matrix-associated laser



Treatment	#	peroxynitrite stock solution (50 mM) vol./add.	No. of add.	Interval
Continuous treatment (CT)				
Continuous treatment	(1)	50 mM x 1 µl	20	30 sec
Continuous infusion	(2)	50 mM x 20 µl	(infusion over 20 min)	
Bolus treatment (BT)	(3)	50 mM x 20 µl	1	-

Chart 1. Preparation of Peroxynitrite-Treated Cytochrome c

dissorption ionization-time of flight mass spectrometry (MALDI-TOFMS), and the mass data were searched for peptide mass finger-printing databases to confirm the appropriate digestion of cytochrome c protein. Furthermore, the digested tryptic peptides were also subjected to a reversed-phase HPLC analysis with a linear gradient of 0 to 45% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid in 60 min, by monitoring with a multi-wavelength detector. The eluted peptide fractions were collected and lyophilized. Each fraction was reconstituted with H_2O/acetonitrile (8 : 2) and analyzed with MALDI-TOFMS. The digested peptide fragments were compared for the observed mass data for each fragment with the calculated mass numbers of the possible tryptic peptide fragments. The tyrosine-, nitrotyrosine-, and tryptophan-containing peptides were compared by the absorption spectra of the peptide fragments obtained from the HPLC analysis with a multi-wavelength detector.

RESULTS

Nitration of Cytochrome c by Continuous Peroxynitrite Infusion *in Vitro* We prepared peroxynitrite-treated cytochrome c in two different ways. One was a continuous infusion of peroxynitrite into cytochrome c in Earle's balanced salt solution (EBSS), and the other was a bolus treatment of cytochrome c with peroxynitrite. Cytochrome c treated with a continuous addition of peroxynitrite solution at 1-min intervals was also prepared. The cumulative final concentration of peroxynitrite was the same in all treatments. In all the preparations of peroxynitrite-treated cytochrome c, tyrosine nitration was confirmed by the detection of 3-nitrotyrosine in the pronase-digested hydrolysates of treated cytochrome c by an HPLC analysis of aromatic amino acid (Fig. 1).

To address the difference in the nitration sites between both nitrated cytochrome c preparations, the continuous and bolus peroxynitrite-treated cytochrome c were subjected to trypsin digestion, HPLC analysis, and MALDI-TOF mass spectrometry. The tryptic peptides of either continuous or bolus peroxynitrite-treated cytochrome c were first resolved by HPLC (Fig. 2). The chromatogram monitoring at 364 nm,

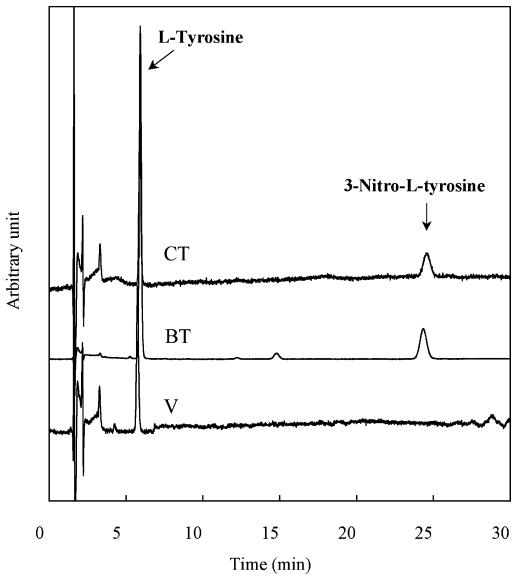


Fig. 1. Detection of Nitrotyrosine in Peroxynitrite-Treated Cytochrome c

Peroxynitrite-treated cytochrome c was hydrolyzed enzymatically, and subjected HPLC analysis for detection of nitrotyrosine. The labels CT, BT, and V on the chromatograms indicate the cytochrome c samples with the continuous, single-dose (bolus), and control (vehicle) treatment of peroxynitrite, respectively.

which is a local absorption maximum wavelength in nitrotyrosine, revealed that 4 peptide fragments contained nitrotyrosine residues in both groups treated with continuous and bolus treatment of peroxynitrite. The chromatogram at 215 nm revealed that the 3 peptide peaks (a, c, i) decreased with peroxynitrite treatment, concomitantly with the increase of the nitrated peptide peaks (b, d, g, j), without any changes in the other tryptic peptides in the chromatogram. Furthermore, from the spectra of these decreasing peaks, it was also confirmed that these peptides contained tyrosine residues. The fractionated tryptic peptides, including tyrosine- and nitrotyrosine-containing peptides, were subjected to a MALDI-TOF mass spectrometer and analyzed with both peptide mass fingerprinting from the public databases and the peptide molecular masses measured. From these results, it was revealed that at least 3 of the 4 tyrosine residues (Y48, Y67, Y74) in

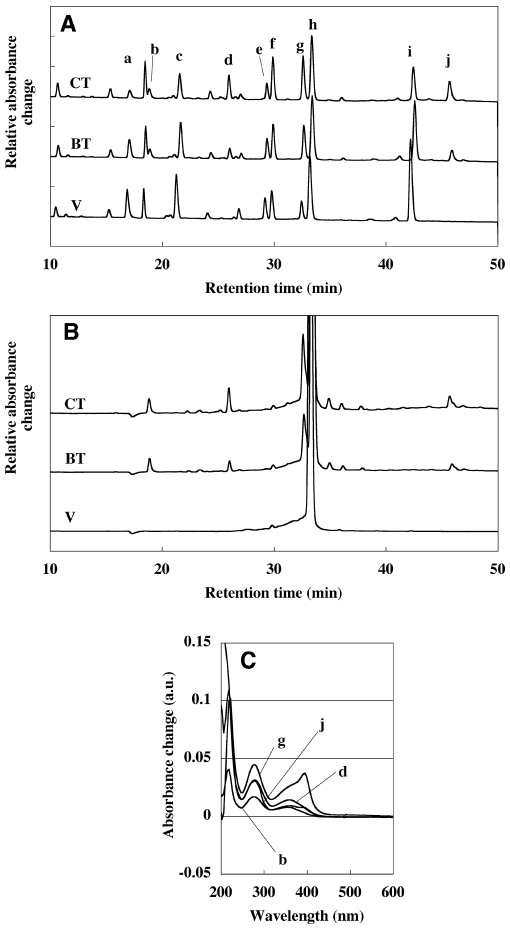


Fig. 2. Chromatogram and Absorption Spectra of the Tryptic Peptides of Peroxynitrite-Exposed Cytochrome c

Cytochrome c treated with peroxynitrite was dialyzed against ammonium carbonate, and digested with a sequencing grade of trypsin for 16 h at 37 °C. The resulting tryptic peptides were separated by HPLC with an ODS column and detected with a multi-wavelength absorption detector. A: chromatograms at 215 nm, B: chromatograms at 364 nm, C: absorption spectra of the nitrated peptide peaks. Small letter alphabetic labels on the peptide peaks and spectra correspond to the labels shown in Table 1. The labels CT, BT, and V on the chromatograms indicate the cytochrome c samples with the continuous, single-dose (bolus), and control (vehicle) treatment of peroxynitrite, respectively.

Table 1. Assignment of Tryptic Peptides Based on the Observed Mass Numbers in Mass Spectrometry

Tryptic peptide	Mass number		Peak label[a)]
	Calculated	Observed	
Y^{74}IPGTK^{79}	677.76	678.08	a
Y^{74}IPGTKMIFAGIK^{86}-NO_2	1483.73	1485.25	d
T^{28}GPNLHGLFGR^{38}	1168.27	1168.56	e or f[b)]
T^{40}GQAPGFSY^{48}TDANK^{53}	1456.44	1456.09	c
T^{40}GQAPGFSY^{48}TDANK^{53}-NO_2	1501.44	1501.44	d
E^{92}DLIAY^{97}LKKATNE^{104}	1507.63	1504.48	g[c)]
I^{9}FVQKCAQCHTVEK^{22}	1633.89	1633.61	h[c)]
G^{56}ITWGEETLMEY^{67}LENPK^{72}	2010.17	2009.56	i
G^{56}ITWGEETLMEY^{67}LENPK^{72}-NO_2	2055.17	2054.06	j

a) The letter indicates the corresponding peak label in the chromatogram shown in Fig. 2. *b*) Peak e and f could not be separately fractionated for the mass measurement. *c*) Assignment of peak g and peak h are also based on their absorption spectra in addition to this peptide mass measurement. Peak h showed a unique absorption around 400 nm based on the heme, which attaches to two cysteine residues of Cys14 and Cys17.

Table 2. Relative Nitrotyrosine Content in Peroxynitrite-Treated Cytochrome c

Treatment	Nitrated cytochrome c (%)[a)]	Fold increase[c)]
Continuous treatment (CT)	37.4±0.37[b)]	1.6
Bolus treatment (BT)	23.4±0.27[b)]	1
Vehicle (V)	0.70[c)]	—

a) Percentage for cytochrome c with 3-nitrotyrosine: Since cytochrome c contains 4 tyrosine residues per molecule, the ratio (%) of nitrated cytochorme c were calculated by dividing the amount of 3-nitrotyrosine by 1/4 amount of total tyrosine. *b*) Mean±S.D. from three independent experiments. *c*) Potency of a continuous treatment for nitration compared with that of a bolus treatment.

Table 3. Relative Nitration of Tyrosine 74 Containing Peptide

Treatment	Relative nitration[a)]
Continuous treatment (CT)	4.25 (4.22, 4.27)[b)]
Bolus treatment (BT)	1 (1.30, 0.70)[b)]
Vehicle (V)	0.03 (0.05, 0.01)[b)]

a) Relative amount of the nitrated peptide containing tyrosine 74 based on the amount of nitration by a bolus treatment. *b*) The value indicates the average of two independent experiments showing individual values in parentheses.

cytochrome c were nitrated without any detectable changes in the other peptide fragments under the conditions of the peroxynitrite treatment in this study (Fig. 2, Table 1). The peptide fragment containing tyrosine 74 (peak a in Fig. 2) was decreased by treatment of peroxynitrite, and the corresponding nitrated peptide (peak d in Fig. 2) was concomitantly increased. Although peak d also contained a certain amount of nitrated peptide containing tyrosine 48 (Table 1), it was found that the corresponding unnitrated fragment (peak c in Fig. 2) was only slightly decreased.

When we focused on the differences between the continuous and bolus peroxynitrite treatment, it was found that nitration at tyrosine 74 was specifically enhanced, and the nitration at tyrosine 67 was also slightly enhanced in cytochrome c with continuous treatment with peroxynitrite. The amount of nitrated cytochrome c (at any tyrosine residue) was 1.6 times larger by continuous treatment than by bolus treatment (Table 2). Whereas, nitration in tyrosine 74 is 4.2 times more frequent in continuously peroxynitrite-treated cytochrome c than in bolus peroxynitrite-treated (Table 3). There were no differences in the nitration at the other tyrosine residues (Fig. 2, Table 1). When peroxynitrite was infused over more than 4 h, cytochrome c nitration was not observed.

***In Vitro* Apoptosis Assay with Peroxynitrite-Exposed Cytochrome c** The ability of tyrosine-nitrated cytochrome c to cause caspase activation was evaluated by an *in vitro* apoptosis assay. Chemically modified cytochrome c was added to an intact cytosolic fraction from C6 cells and incubated at 30 °C for 90 min. For the detection of caspase activation, cleaved caspase-3 fragments (p17, p19) were observed *via* immunoblotting analysis with the specific antibody for the cleaved fragments of caspase-3. With the nitrated cytochrome c that had been continuously treated with peroxynitrite, caspase activation was not observed in the assay, while the activation was observed when the control and the intact cytochrome c preparations were used. With the TNM-treated cytochrome c, caspase activation was also attenuated (Figs. 2, 3). Interestingly, caspase activation was observed with the cytochrome c preparation treated with a bolus (1 mM) of peroxynitrite (Fig. 3). The activation was also observed with cytochrome c treated with a single low dose (50 μM) of perox-

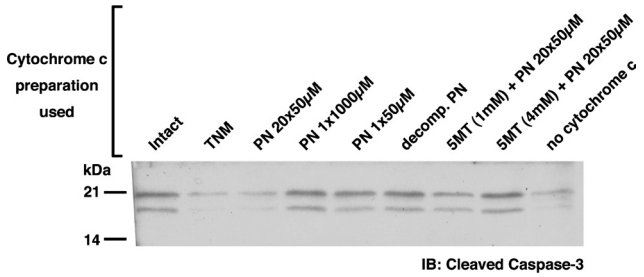


Fig. 3. Detection of Caspase-3 Fragments after *in Vitro* Caspase Activation Reaction with Exogenous Cytochrome c Nitrated by Continuous Peroxynitrite Treatment

Cytosolic fraction prepared from the intact cells was incubated with the cytochrome c preparations independently pretreated as indicated on the top of each lane. Caspase-3 active fragments (p17, p19) were observed with the specific antibody using chemiluminescence detection. TNM: tetranitromethane, PN: peroxynitrite, decomp. PN: decomposed PN, 5MT: 5-methoxytryptamine. PN 20×50 μM indicates 20 times of continuous treatment with 50 μM peroxynitrite. Representative data of the same results from three independent experiments are shown.

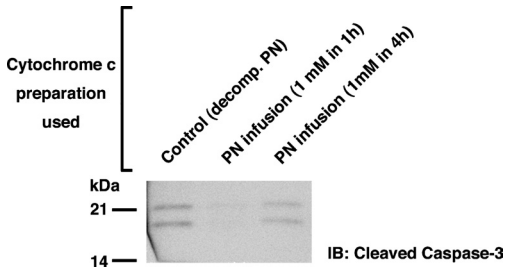


Fig. 4. Detection of Caspase-3 Fragments after the *in Vitro* Caspase Activation Reaction with the Exogenous Cytochrome c Nitrated by Continuous Peroxynitrite Exposure

Cytosolic fraction prepared from the intact cells was incubated with the cytochrome c preparations independently pretreated with peroxynitrite as indicated. The caspase-3 active fragments (p17, p19) were observed in the same way as in Fig. 3. PN: peroxynitrite, decomp. PN: decomposed PN, "PN 1 mM in 1 h" indicates that peroxynitrite was infused into the cytochrome c solution for 1 h, and the total cumulative concentration of peroxynitrite was 1 mM.

ynitrite. From these results, it was found that the ability of cytochrome c to induce the caspase cascade activation was attenuated by continuous or repeated peroxynitrite exposure, but not by a bolus treatment. For further investigation regarding whether this functional loss with continuous treatment of peroxynitrite depends on the nitration of cytochrome c or not, a nitration-specific peroxynitrite scavenger, 5-methoxytryptamine (5MT), was employed. 5MT has been found to inhibit tyrosine nitration by peroxynitrite without affecting the tyrosine-oxidizing activity.[28] In the presence of 5MT, cytochrome c was treated with peroxynitrite repeatedly, and this cytochrome c preparation was then subjected to an *in vitro* apoptosis assay. The result indicated that simultaneous 5MT treatment preserved the ability of cytochrome c to induce caspase activation with continuous peroxynitrite exposure (Fig. 4). Together with the results from the *in vitro* apoptosis assay, it was found that continuous exposure of peroxynitrite caused the nitration of cytochrome c and the attenuation of its ability for caspase activation. However, a bolus of peroxynitrite exposure did not show this effect, even though the treated cytochrome c was nitrated.

DISCUSSION

From the *in vitro* apoptosis assay, it was found that caspase cascade activation was suppressed when cytochrome c was treated with continuous peroxynitrite exposure, but this effect was not observed with a bolus treatment of peroxynitrite. We suggest that the attenuation of cytochrome c activity for caspase activation was closely related to tyrosine nitration because 5MT, a nitration-specific scavenger, preserved the activity of cytochrome c (Fig. 3). Our results from a bolus treatment of cytochrome c with peroxynitrite is consistent with a previous report,[24] in which cytochrome c treated with a bolus of peroxynitrite did not lose its ability for caspase cascade activation.

From the analysis of peroxynitrite-treated cytochrome c, it was found that all 4 positions of the tyrosine residues were nitrated in both methods of treatment. However, the tyrosine nitration at position 74 was increased when cytochrome c was exposed to continuous peroxynitrite treatment, compared with a bolus of peroxynitrite (Fig. 2, Table 1), although other tyrosine residues were nitrated to a similar extent with both treatments. By continuous treatment with peroxynitrite, the total amount of nitration to cytochrome c increased in some extent, but tyrosine 74 is more specifically nitrated by continuous treatment than by bolus treatment (Tables 2, 3). This suggests that continuous exposure of peroxynitrite is not only different from bolus one in the total amount of nitration, but also in the specificity of nitration. Based on the HPLC and MALDI-TOFMS analyses of the tryptic peptides of peroxynitrite-treated cytochrome c, methionine oxidation and modifications other than tyrosine nitration were not observed.

Our results suggest that the differential effect of continuous peroxynitrite exposure to cytochrome c on its potency for caspase activation depends on the extent of the nitration at tyrosine 74.

The tyrosine 74 residue is known to be important for the electron transfer reaction in mitochondria[29,30] and is located near the tyrosine 67 residue, which is the primary target for bolus peroxynitrite treatment.[22] Cytochrome c exposed to a low dose of peroxynitrite impaired electron transfer ability, as shown in our previous study.[23]

From the spectral analysis of nitrated cytochrome c, there was almost no difference between the nitrated and control cytochrome c except a slight (less than 2 nm) shift of the Soret band. This band shift may reflect a slight change of the coordination conditions. Additionally, HPLC and MALDI-TOFMS analyses also suggest that there are no other detectable changes in the tryptic digest of the protein, except nitration of 3 tyrosine residues. Based on these observations, the differences between these 2 nitrated cytochrome c preparations can be attributed to the enhanced nitration of a specific tyrosine residue. These results implicate that the pattern of peroxynitrite exposure (continuous or bolus) results in different modifications at a specific tyrosine residue, and finally in different effects on caspase cascade activity. The redox status of cytochrome c is known to be involved in caspase activation. In this study, all the cytochrome c preparations, including the controls, were used in the oxidized form, as confirmed by the measurement of the absorption spectra of the samples. In this study, the mechanism of cytochrome c inactivation by peroxynitrite treatment was not fully revealed yet. It is known that cytochorme c interacts with apoptotic protease activating factor-1 (Apaf-1). It is possible that enhancement of Tyr 74 ni-

tration in cytochrome c may reduce its interaction with Apaf-1 or increase its dominant negative property. Although the molecular mechanisms of apoptosome inactivation by the specific nitration of cytochrome c still need to be clarified, our findings suggest that the nitration status at specific tyrosine residues can affect the ability of cytochrome c to cause capsase activation. It is known that cytochrome c interacts with Apaf-1 to form an active apoptosome complex. The enhanced nitration at tyrosine 74 may affect active apoptosome formation.

In conclusion, this study determined that cytochrome c nitrated by continuous treatment with peroxynitrite lost its ability to cause caspase cascade activation *in vitro*, whereas cytochrome c nitrated by a bolus peroxynitrite treatment had preserved activity. The differential property of the continuously peroxynitrite-exposed cytochrome c was closely related to the enhanced nitration of specific tyrosine residues. This is the first study to demonstrate that protein nitration at specific residues affects caspase activation. We suggest that the exposure pattern of peroxynitrite, including the duration and concentration, is important for cytochrome c activity for caspase activation.

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