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Reduction of the nitro group during sample preparation may cause underestimation of the nitration level in 3-nitrotyrosine immunoblotting☆

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Abstract

We noted differences in the antibody response to 3-nitrotyrosine (NO_2Tyr) in fixed and non-fixed tissues, and studied therefore potential problems associated with non-fixed tissues in Western blot analyses. Three different monoclonal anti-nitrotyrosine antibodies in Western blot analysis of inflammatory stimulated rat abdominal, liver and lung tissue homogenates caused no immunoreactivity, in contrast to a polyclonal nitrotyrosine antibody applied in fixed and non-fixed tissues. Western blot studies using both mono- and polyclonal antibodies showed a temperature- and heme group-dependent reduction of NO_2Tyr in nitrated rat and bovine serum albumin incubated with dithiothreitol. Mass spectrometric analyses of a nitrated peptide angiotensin II revealed under similar conditions a positive temperature effect between 56 and 70 °C on reduction of NO_2Tyr to 3-aminotyrosine which is not detected by anti-NO_2Tyr antibodies. Western blot analysis may therefore underestimate the level of tissue nitration, and factors causing a reduction of NO_2Tyr during sample preparation might conceal the actual nitration of proteins.

Keywords: Angiotensin II; Mass spectrometry; 3-Nitrotyrosine; Temperature; Western blot

1. Introduction

Tyrosine nitration has been observed in various inflammatory conditions and has been linked to the presence of reactive nitrogen species (RNS) and reactive oxygen species (ROS). Initially, the nitration product, 3-nitrotyrosine (NO_2Tyr), was believed to be a marker of the reaction between peroxynitrite and tyrosine, but recent studies have recognized the involvement of nitrite and peroxidase enzymes in the nitration mechanism as well [1]. Understanding the protein nitration aspects of functional and regulatory mechanisms requires protein identification. Western blot studies have often identified one major protein to be nitrated [2–5], although it is possible that other less abundant or insoluble proteins are also nitrated. Several reports have described "denitrase" activity in the lung, spleen and heart using Western blot analysis [6–9]. Neither an enzyme catalyzing the reaction, nor a product of this reaction was identified. Recently, however, mitochondrial metabolic pathways were found to be influenced by reversible nitration [10].

From many in vitro experiments, it is apparent that the nitration of a single tyrosine residue in proteins may alter their enzyme function and thereby contributes to the pathogenesis of diseases [11,12]. The formation of NO_2Tyr has been demonstrated in many pathological conditions, such as pulmonary [13] and heart disease [4,14,15], atherosclerosis [2], systemic sclerosis [16], Parkinson's disease [17] and Alzheimer's disease [18,19]. A number of experiments have demonstrated in vivo nitration and each study has listed a limited number of

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nitrated proteins involved in each disease [2,4,17,20–22]. However, from these NO_2Tyr-containing proteins alone, it is difficult to identify pathophysiological consequences regarding specific disease development. Most studies demonstrating specific in vivo nitration have utilized immunochemistry. However, Aslan et al. [23] demonstrated the nitration of a specific peptide (actin) with mass spectrometry (MS) after protein separation with 2D electrophoresis. Further, Park et al. [24] found by MS specific nitration on p65 which inactivated NF-kappaB activity. MS is a useful tool for determining the structure of the post-translational modification. Prior to MS analysis, the sample preparation may include immunochemistry with gel electrophoresis (1D or 2D), hydrolysis and the in-gel digestion of proteins, all of which being demanding procedures for protein stability and chemical exertion. Furthermore, some problems involved in immunochemistry have been demonstrated in terms of reduction of the nitro group by interference from thiols and heme-containing proteins [6–8].

Reduction of the nitro group, i.e. conversion of NO_2Tyr to aminotyrosine (NH_2Tyr), may lead to failure of an antibody to recognize the modified tyrosine. Such a problem may be enhanced by the regular use of dithiothreitol (DTT) and high temperature in Western blot to disrupt protein disulfide bonds and expose hidden sites to antibody interaction. DTT is a reducing agent and high temperature speeds up chemical reactions. In the present study, we wanted to evaluate the heme protein interference with NO_2Tyr in nitrated proteins, as well as the effects of DTT and temperature. Mixtures of nitrated albumin and nitrated angiotensin II (ATII) were incubated with heme proteins and DTT at different temperatures, and the products were analysed by HPLC, Western blotting and MALDI-TOF MS, nano electrospray MS (NanoESI/MS) and NanoESI/MS/MS, and MALDI-TOF/TOF. Free 3-nitrotyrosine is not part of this paper. However, since we recently obtained by GC–MS/MS [25], in vivo data on successively increasing circulating levels of 3-nitrotyrosine up to 8 days after i.p. injection of zymosan, we examined by immunochemistry in the present study the degree of 3-nitrotyrosine expression in liver and lung homogenate from these animals. We also investigated the nitrotyrosine blot immunoreactivity of liver homogenate for both monoclonal and polyclonal nitrotyrosine antibodies.

2. Experimental

2.1. Materials and chemicals

Phenylmethylsulphonyl fluoride, trypsin inhibitor, leupeptin, antipain, chymostatin, bicarbonate, Tween 20 and pepstatin and zymosan were obtained from Sigma Chemical Co (St. Louis, MO, USA). HEPES, sodium hydrosulphite (dithionite), magnesium chloride, sodium chloride and EDTA were purchased from Merck (Darmstadt, Germany). Sodium dihydrogenphosphate monohydrate and disodium hydrogenphosphate dihydrate were purchased from Scharlau (Barcelona, Spain). BCA protein assay reagent was obtained from Pierce (Rockford, IL, USA). NuPAGE LDS sample buffer, NuPAGE 7% Tris–acetate gels and NuPage 4–12% Bis–Tris & Tris–acetate, MES & MOPS Running buffer and NuPage Transfer buffer were obtained from Novex (San Diego, CA, USA). The chemiluminescent detection system model Western-Star were obtained from Tropix (Bedford, MA, USA).

2.2. Zymosan-induced inflammation

Zymosan (50 mg/ml in 0.9% NaCl) solution was heated at 95 °C for 30 min prior to intraperitoneal (i.p.) injection [26]. Twenty-four and 192 h after the bolus injection, the rats were anaesthetised by an i.p. injection of 0.4 ml mixture of ketamine hydrochloride (10 mg/ml, Ketalar, Parke-Davis, Barcelona, Spain) and xylazine hydrochloride (20 mg/ml, Rompun vet, Bayer Corp., Leverkusen, Germany) solution diluted in 0.4 ml phosphate buffer saline (PBS, pH 7.2) followed by isoflurane (Baxter Medical AB, Kista, Sweden) inhalation. Abdominal exsudate was withdrawn. The blood was acutely withdrawn by puncture of the left ventricle after opening of the chest. Immediately thereafter, the organs of the animals were perfused via the left ventricle in situ with PBS until the liver was visually free from blood. The organs were cut out and snap frozen in liquid nitrogen and stored at −80 °C.

2.3. Histology-immunohistochemistry (fixed tissue)

Tissue specimens were immersed in phosphate-buffered 4% paraformaldehyde (pH 7.0) for 24 h, cryoprotected in sucrose for 36 h and frozen in isopentane-dry ice at −80 °C. Sections of about 6 μm were cut by a cryostat and were either stained with hematoxylin–eosin for routine examination, or subjected to immunohistochemistry according to the following description. Prior to immunohistochemistry, endogenous peroxidase was blocked by 0.3 vol.% H_2O_2 in methanol. The tissues were incubated with a polyclonal rabbit anti-NO_2Tyr antibody (Upstate Biotechnology, Lake Placid, NY, USA), diluted (1:300) in 1% BSA in PBS and incubated at room temperature for 60 min. The sections were then incubated with DAKO-Envision (Dakopatts, Stockholm, Sweden) for 30 min. Positive immunoreactivity was visualized with 3-diaminobenzidine tetrahydrochloride (DAB, Dakopatts), producing a brown stain, and the sections were counterstained with Mayer's hematoxylin. The specificity of the anti-NO_2Tyr antibody was tested by pre-incubation with 3-NO_2Tyr (Sigma–Aldrich, St. Louis, MO, USA) or by the omission of the primary antibody; neither resulted in any brown staining of the tissues.

2.4. Nitration of albumin and angiotensin II

Bovine and rat albumin, as well as ATII (all from Sigma–Aldrich, St. Louis, MO, USA) were nitrated as we previously described [25,27], using a 50-fold molar excess of tetranitromethane (TNM) over night at 4 °C. The modified albumin was rapidly desalted using PD-10 columns (Pharma Biotech, Sweden). Further purification and pre-concentration was performed by ultrafiltration three times in HEPES buffer at pH 7.4 using a Vivaspin 500 cartridge, cutoff 30 kDa, Vivascience (Lincoln, UK). Quantification of nitrated protein was

performed by a BCA protein assay (Pierce Chemical, Rockford, IL, USA).

2.5. Protein extraction from tissues

Tissue specimens from liver and lung as well as abdominal inflamed tissue/exudate were homogenized with an Ultra-Tarrax T8, IKA Labortechnik (Staufen, Germany) in five volumes of ice-cold homogenisation buffer (25 mM HEPES at pH 7.4) containing 0.1 mM EDTA, and 0.01 mg/ml of each of the following compounds: phenylmethylsulphonyl fluoride, trypsin inhibitor, leupeptin, antipain, chymostatin and pepstatin. The homogenates were centrifuged at 10,000 × *g* for 45 min, the protein concentration of the supernatant was determined by the BCA protein assay reagent (see above).

2.6. Western blot analysis

The Western blot protocol of liver, lung and inflammatory homogenate from zymosan-treated rats followed the instructions issued by Novex (Invitrogen, San Diego, CA, USA). The homogenate and nitrated albumin were heated to 70 °C for 10 min in a reducing sample buffer. The proteins were fractioned by NuPAGE 7% Tris–aetate gels, 4–12% Bis–Tris gel and electroblotted to a polyvinyldiene fluoride (PVDF) membrane (BioRad Hercules, CA, USA). In addition, the PVDF membrane was incubated with 0.2% casein and 0.3% Tween 20 in PBS for one hour at room temperature to block non-specific binding sites. The PVDF membrane was incubated over night with anti-NO_2Tyr antibody (Upstate) at 4 °C. In similar experiments, we compared different anti-NO_2Tyr antibodies (Upstate, Cayman Chemicals and a gift from Dr. L.L. Ng). The blot was washed three times and then incubated for 1 h with the secondary antibody. After being washed five times, the blot was visualized with a chemiluminescence detection system that utilizes enzyme-linked immunodetection. The chemiluminescence reaction was detected using hyperfilm ECL from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.7. Controls for NO_2Tyr antibody binding

Specificity of the anti-NO_2Tyr antibody was confirmed by: (i) omission of the primary antibody, (ii) chemical reduction of NO_2Tyr-residues with dithionite (20 mM dithionite in 50 mM of sodium bicarbonate buffer, pH 9, 1 min), or (iii) pre-incubation of the primary antibody with 10 mM of 3-NO_2Tyr for 1 h at room temperature. We have previously demonstrated that the monoclonal antibody (Upstate) has no cross-reactivity with native BSA under similar experimental conditions [27].

2.8. Sample mixture of nitrated rat serum albumin/DTT/myoglobin for Western blot

Nitrated rat serum albumin was mixed with myoglobin (Fe^{3+}) and incubated at ambient temperature for 30 min. Prior to gel loading, the samples were diluted with sample buffer, and heated in a water bath at 90 °C, 70 °C, 56 °C or room temperature for 10 min, with or without the addition of DTT (NuPAGE® Reducing Agent). The normal Western blot procedure then followed.

2.9. Reverse-phase HPLC purification and analysis of NO_2Tyr-ATII incubated with DTT and heme proteins

The HPLC system consisted of two LKB 2150 HPLC pumps (Pharmacia AB, Uppsala, Sweden), and a Model 2158 Uvicord SD UV-detector. The mobile phase consisted of solvent A: 0.1 vol% TFA in water and solvent B: 0.06% (v/v) TFA in 90% acetonitrile, 9.9% water. The gradient was run from 10% to 40% solvent B in 30 min and up to 80% solvent B in 5 min. Elution peaks were monitored at 214, 280 and 350 nm. Samples of 50 μl, acidified by TFA, were introduced via an injector onto a Jupiter C-4 analytical column (250 × 4.6 mm i.d., pore size 300 Å) preconditioned with 0.1 vol.% TFA. The flow rate was 0.85 ml/min. Fractions of interest were concentrated using a Speed-Vac instrument and the samples were dried under vacuum at ambient temperature. Residues were dissolved in 50 vol.% methanol containing 1 vol.% formic acid prior to MS analysis.

2.10. Analysis of a sample mixture of NO_2Tyr-ATII/DTT/Hb and myoglobin

Quantification was carried out by acid hydrolysis in 6 M HCl followed by amino acid analysis on a Biochrom 30 amino acid analyzer. An amount of 0.8 μg of HPLC-purified NO_2Tyr-ATII, was incubated with 2.5 nmol of myoglobin or 2.5 nmol of Hb and 1 μmol of DTT in a final volume of 100 μl of PBS (pH 7.4) to obtain a solution of 7.3 μM NO_2Tyr-ATII, 25 μM Hb and 10 mM DTT. The mixture was boiled for 10 min. After cooling to room temperature, a 25-μl aliquot was acidified with HPLC mobile solvent A and injected. The corresponding fractions were collected; NO_2Tyr-ATII was eluted after using 30% of solvent B.

2.11. MALDI-TOF MS analysis

Samples consisting of Hb, DTT and the nitrated peptide were desalted and concentrated prior to the MALDI-TOF MS analysis according to a procedure described previously [28]. After sample loading onto the column, followed by a washing step, the analytes were eluted with 50 to 100 nl of matrix solution {α -cyano-4-hydroxycinnamic acid (Sigma), 20 μg/μl in acetonitrile–0.1 vol.% TFA (70:30, v/v)} directly onto the MALDI target. Positive ions were recorded using a Bruker-Reflex mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) in the reflector mode, using delayed ion extraction (approx. 350 ns delay). Ions below *m/z* 600 were deflected. A MALDI-TOF/TOF instrument (4700 Proteomics Analyzer with TOF/TOF optics, from Applied Biosystems) was used for zymosan exudate in-gel digested samples. Obtained mass spectra were then used for protein identification in the Mascot on-line database (http://www.matrixscience.com). The external calibration of tryptic peptide mixtures was achieved

using the monoisotopic masses of tryptic lactoglobulin peaks at *m/z* 842.5094 and 2211.1046. The results from the Mascot search program are reported as probability based scores, i.e. scores are reported as $-10\log_{10}(P)$, where P is the absolute probability that the observed match is a random event. The score corresponding to a probability value of 0.05 is reported by default.

2.12. Nanoelectrospray MS analysis

NanoESI/MS and NanoESI/MS/MS were performed on a quadrupole time-of-flight mass spectrometer (QTOF, Micromass, UK). A 10-μl aliquot of the sample containing heme, DTT and NO_2Tyr-ATII was loaded onto a microcolumn, the column was washed and then analytes were eluted directly into the nanoelectrospray needle (Protana, Odense, Denmark) using a 2-μl aliquot of the spray solution.

3. Results

3.1. Immunohistochemistry

Morphological examination with regard to inflammatory alterations of the tissues under investigation, as seen by routine staining with hematoxylin and eosin showed no obvious difference between control tissues (i.e., liver and lung) and tissues from zymosan treatment for 24 h. It should be pointed out that minor changes in the tissues in response to the treatment may be concealed by the fact that cryostat section of the tissues (a necessity for the antibody used) causes some morphological derangements, a priori. In any case, no inflammatory reaction was evident in either tissue after zymosan treatment. Interestingly, both normal liver and lung expressed positive immunoreactivity to protein nitrotyrosine, localized in the hepatocytes and in the alveolar walls respectively. Tissues from animals treated with zymosan for 24 h displayed no clear difference from the tissue of controls with regard to such immunoreactivity (Fig. 1).

3.2. Western blot and mass spectrometric analysis

The specificity of the Upstate monoclonal antibody for nitrated proteins was established by experiments performed on nitrated serum albumin as a positive control. The negative control was performed using native BSA, by reducing the nitro groups of the blotted proteins with dithionite or blocking the antibody interaction with NO_2Tyr. When we excluded the primary antibody to evaluate the impact of the second antibody, the blot showed no band corresponding to nitrated albumin (not shown).

To evaluate tissues with expected high concentration of protein-associated NO_2Tyr, we analyzed by gel electrophoresis and Western blotting the abdominal exudate from zymosan-treated rats (Fig. 2). The exudate proteins were separated on NuPAGE Tris–acetate or Bis–Tris gel and half the gel was stained with Coomassie blue for further MS analysis. The other half was blot-immunoreacted with monoclonal mouse anti-NO_2Tyr IgG. The PVDF membrane was also incubated with dithionite to exclude or prove secondary antibody cross-reactions (Fig. 2B). A few studies have reported nitrated albumin in inflammatory conditions, [5,29], and we therefore excised and investigated the bands around 60 kDa and some additional bands that were strongly stained, but not in line with the secondary antibody cross-reactions (Fig. 2A, band #1–5). The bands were in-gel digested by trypsin and analyzed with a MALDI-TOF/TOF instrument. Proteins which scored (see Section 2.11) more than 53 in the MASCOT searches were significant at the $P < 0.05$ level. The result from gel band #1 revealed that the peptide data correlated to C-reactive protein, MW 25737 Da, from rat with a score of 213 ($P < 5 \times 10^{-8}$). Bands #2, #3 and #5 were all assigned to rat albumin, MW 68724 Da), with scores higher than 53 ($P < 0.05$). The appearance of albumin as three different molecular bands is likely due to the degradation of nitrated albumin. Band #4 was assigned to hemopexin, MW 51277 Da with a score of 67 ($P < 0.002$). In each of the mass spectra obtained from the individual bands, we searched for a change of the nitration

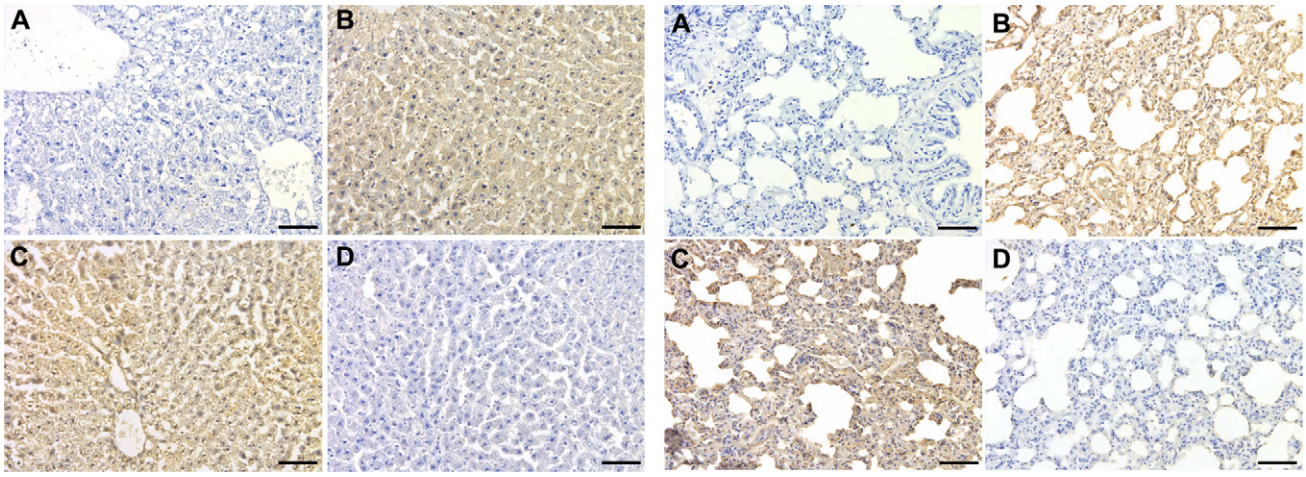

Fig. 1. Immunohistochemical demonstration of nitrotyrosine expression (brown) of rat liver (left panel) or rat lung (right panel) after 24 h subsequent to NaCl (A, B, and D) or zymosan administration (C). In (A), the primary antibody was omitted. In (D), the antibody was preincubated with 10 mM of 3-nitrotyrosine as a control of antibody specificity. Bar is 50 μm.

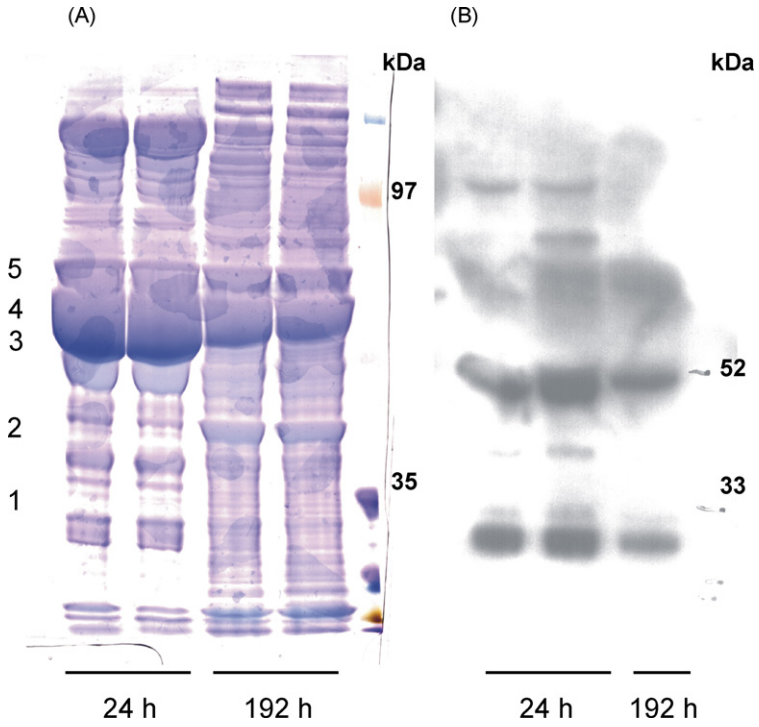

Fig. 2. NuPAGE separation, visualized by Coomassie blue staining without use of antibodies (A) and Western blot analysis with monoclonal anti-nitrotyrosine IgG (B) of abdominal exudate homogenate of zymosan-treated rats after 24 and 192 h. In panel (A), lane 1: the bands # 1–5 were cut out, in-gel digested and analyzed on MALDI-TOF/TOF. The bands around 52 kDa were considered cross reactions to the secondary antibody, and were thus not cut out.

pattern, i.e. for a decrease by 30 Da in the protonated molecules (i.e., $[M+H]^+$) [27], that would suggest conversion of a nitro group (46 Da) to an amino group (16 Da) [30]. No such pattern was found in the mass spectra of the individual gel bands (data not shown).

3.3. Immunoreactivity using anti-NO_2Tyr IgG

In an in vitro experiment, we tried to evaluate the degradation level of the nitrated albumin in gel electrophoresis under normal reducing conditions (Fig. 3A). The gel electrophoresis was performed using two NuPAGE 4–12% Bis–Tris gel, where we separated a mixture of nitrated albumin molecules, with or without the addition of heme-containing protein (i.e., myoglobin), and DTT to evaluate their influences on the reduction of the nitro group. The duplicate gel was transferred to a PDVF membrane for blot immunoreactivity using anti-nitrotyrosine IgG to evaluate a possible reduction response influenced by either DTT or myoglobin or a combination of both. Fig. 3B shows Western blotting under reducing conditions at four different temperatures with and without myoglobin and DTT. The staining of bands, corresponding to nitrated albumin, incubated with DTT and myoglobin at temperatures of 56, 70 and 90 °C were markedly fainter than the nitrated albumin without myoglobin (lane #1 compared with lane #3). In contrast, at room temperature, the staining intensity of the nitrated albumin, incubated with myoglobin, was not altered (bottom left, lane #1 compared with lane #3). Without myoglobin (lane #3 and #4), DTT increased immunostaining at all temperatures. With myoglobin (lane #1 and #2), DTT increased immunostaining at room temperature, made little difference at 56 °C and reduced immunostaining at 70–90 °C Unexpectedly, without the addition of DTT, we noted that the proteins ran more rapidly in the gel. The conditions were the same for the polyclonal antibody (not shown) and the result was also the same.

Having noted the differences with temperature, we also investigated the nitrotyrosine blot immunoreactivity of liver homogenate at room temperature for both polyclonal and monoclonal antibodies (Fig. 3C and D, respectively). Nitrated rat serum albumin (nRSA) was used as a positive control. The control experiment for nitrotyrosine immunoreactivity was performed by preincubating the blot in 20 mM of dithionite (Fig. 3C and D, left panels). The immunoreactivity to NO_2Tyr with polyclonal antibody was positive and the reaction disappeared with dithionite treatment. In contrast, no immunoreactivity to nitrotyrosine of liver proteins was seen with the monoclonal antibody (Fig. 3D).

3.4. HPLC and mass spectrometry of NO_2Tyr-ATII

We performed an experiment for NO_2Tyr-AII, with and without the addition of heme-containing protein (Hb or myoglobin) and DTT, as described by Kamisaki et al. [8], and used mass spectrometry, see below, to determine the modified structure of the peptide. The peptide solutions were incubated with either DTT or Hb, and the mixtures were heated at different temperatures. The isolation methods were either RP-HPLC (Fig. 4, top) with manual collection of fractions of reaction products, or POROS microcolumns [28] to remove salts and proteins prior to subject the samples to MS analysis. The individual

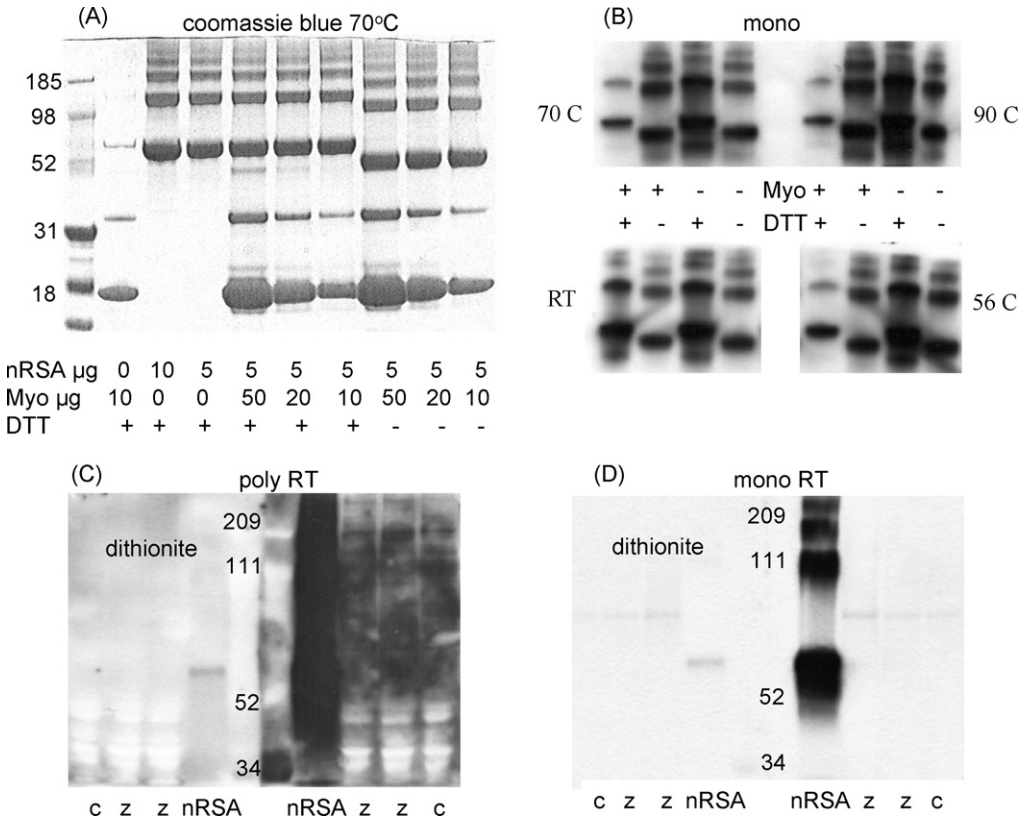

Fig. 3. NuPAGE separation of nitrated rat serum albumin (nRSA) incubated with and without myoglobin under reducing conditions. (A) nRSA at 70 °C, visualized on gel (4–12% Tris–acetate) by coomassie blue staining. (B) Western blot with anti-nitrotyrosine IgG (monoclonal, Upstate) at four different temperatures, i.e. RT, 56 °C, 70 °C and 90 °C. Western blot at RT, using polyclonal (C) and monoclonal antibody (D), of nRSA (positive control) and rat liver homogenate (c, control; z, 192 h after i.p. injection of zymosan). Left panel in both (C) and (D): reversed samples after dithionite treatment. RT, room temperature; DTT, dithiothreitol.

HPLC chromatograms shown in Fig. 4 were obtained from: (ii) NO_2Tyr-ATII, (iii) a mixture of NO_2Tyr-ATII and Hb, (iv) a mixture of NO_2Tyr-ATII and DTT, and (i and v) a mixture of NO_2Tyr-ATII/DTT/Hb. All these mixtures were heated at 90 °C for 10 min. The mixture of NO_2Tyr-ATII/DTT/Hb caused additional HPLC peaks compared with mixtures in which one of the parameters had been excluded, indicating a change in structure. Of these peaks, the peak from sample (i) eluting at 10.56 min was examined with NanoESI-QTOF and the MS spectrum with m/z 531.5 $[M+2H]^{2+}$ is in accordance with the aminotyrosin-

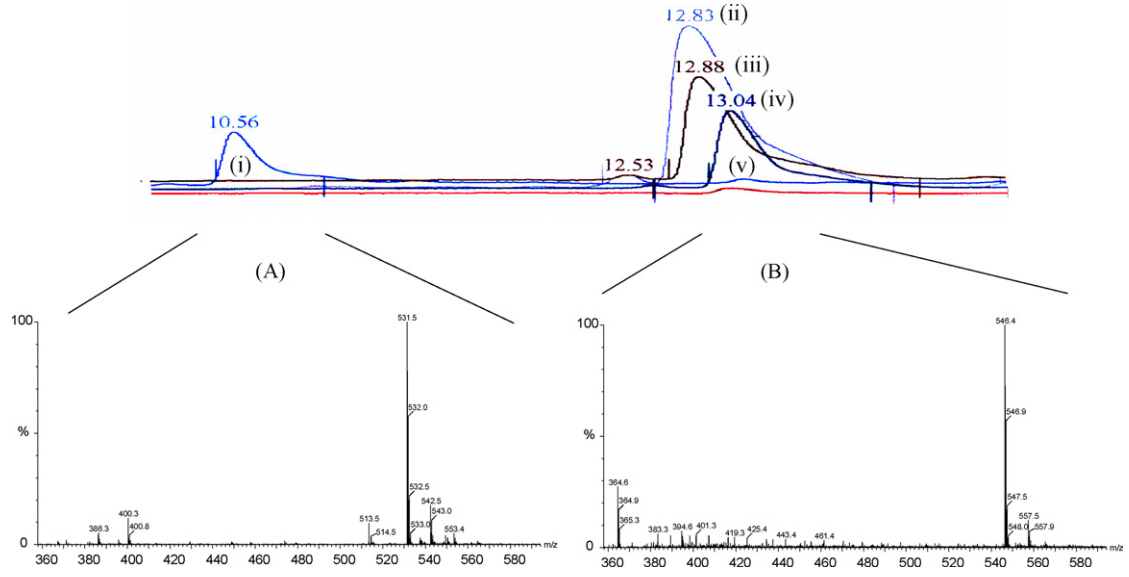

Fig. 4. Upper panel shows RP-HPLC chromatograms of NO_2Tyr-ATII incubated at 90 °C for 5 min with no reducing agent (ii), with Hb (iii), with DTT (iv) or with Hb + DTT (i + v). The NanoESI–MS mass spectra of the peaks eluting at 10.56 and 13.04 min are shown in the lower panel (A) from sample (i) and in panel (B) from sample (v), respectively.

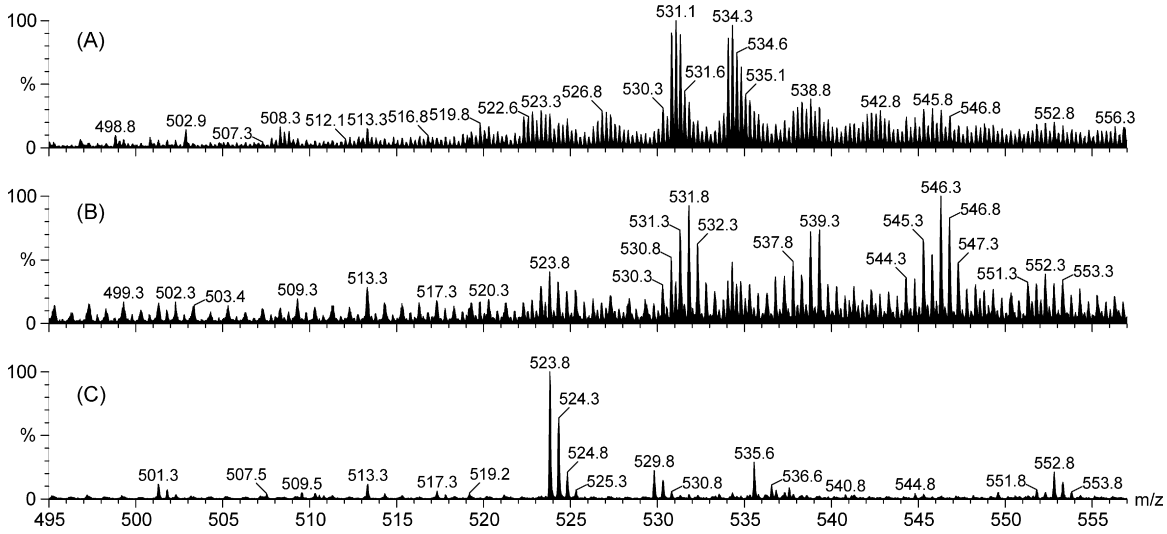

Fig. 5. NanoESI–MS identification of angiotensin II (ATII) and nitrated angiotensin II (NO_2Tyr-ATII). NO_2Tyr-ATII was mixed with myoglobin and DTT (A), and with myoglobin alone (B). As a control non-nitrated ATII was mixed with myoglobin and DTT (C). All samples were heated to 90 °C in a water bath for 10 min. After heating, salts and myoglobin were removed using microcolumns (poros 20, R2), and samples were analyzed by NanoESI-QTOF MS. Note: As NanoESI–MS yields doubly charged ions, e.g. $[M+2H]^{2+}$, m/z values are half of those obtained by MALDI-TOF (see Fig. 6).

containing ATII, i.e., NH_2Tyr-ATII, (Fig. 4, bottom, A). The mass spectrum of the second HPLC peak eluting at 12.53 min in the same run contained mass fragments that could not be assigned (data not shown). The third collected HPLC peak from sample (v) eluting at 13.04 min contained NO_2Tyr-ATII, m/z 546.5 $[M+2H]^{2+}$ (Fig. 4, bottom, B). The areas of the HPLC peaks of NH_2Tyr-ATII and NO_2Tyr-ATII have not been used for quantitative analysis due to the different UV absorbance maxima and unknown molar absorptivities of these compounds.

3.5. Mass spectrometry

In Figs. 5–7, the MS results are shown for NO_2Tyr-ATII after incubation at different temperatures of the peptide with heme-containing protein (myoglobin or Hb) with and without DTT, or with DTT alone. The NanoESI–MS spectrum of NO_2Tyr-ATII incubated with myoglobin and DTT shows an ion at m/z 531 which corresponds to NH_2Tyr-ATII $[M+2H]^{2+}$, whereas an ion at m/z 546 corresponding to $[M+2H]^{2+}$ of NO_2Tyr-ATII

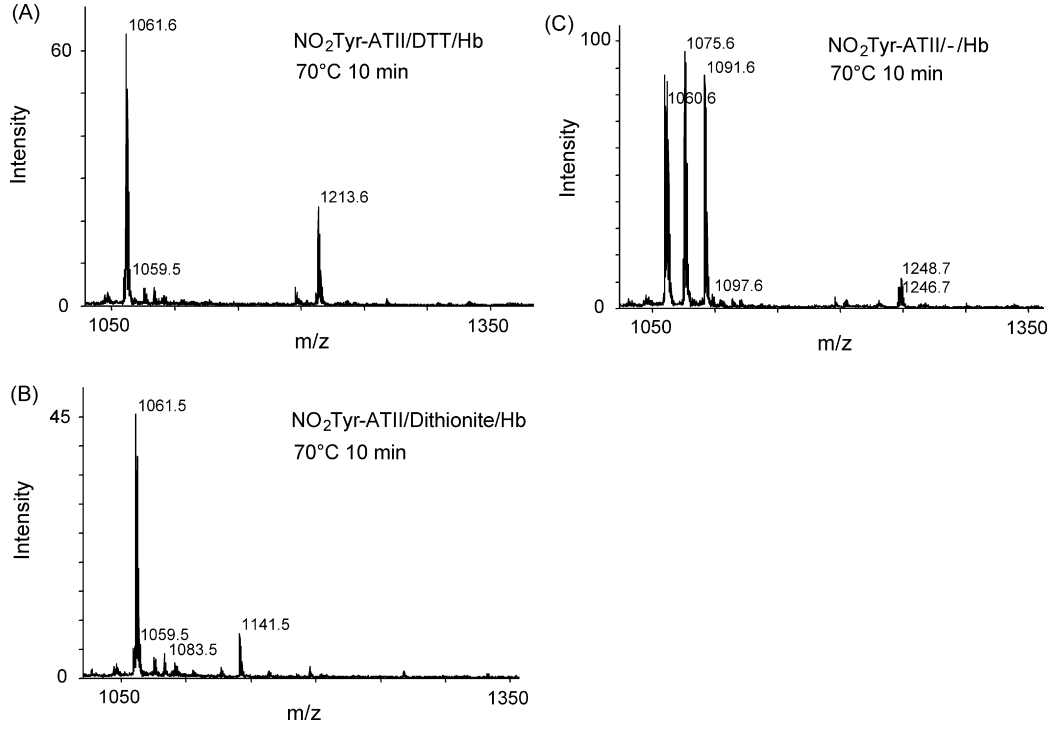

Fig. 6. MALDI-TOF mass spectra of NO_2TyrATII incubated with DTT and Hb (A), dithionite and Hb (B), and with Hb alone (C) at 70 °C for 10 min each.

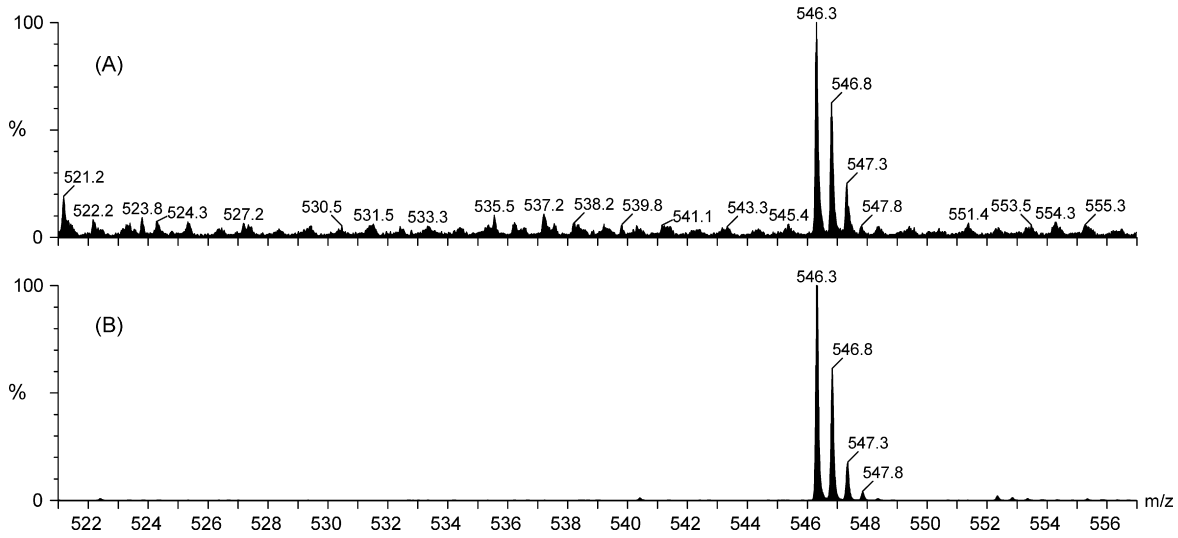

Fig. 7. NanoESI mass spectra of NO_2Tyr-ATII incubated with DTT and Hb (A) or DTT (B) at 56 °C for 45 min each. See comment in the legend to Fig. 5.

is missing (Fig. 5A). This means that at 90 °C myoglobin and DTT reduce NO_2Tyr-ATII to NH_2Tyr-ATII almost completely, while myoglobin without DTT causes a partial reduction as both ions *m/z* 546 and *m/z* 531 are present at comparable intensity in the mass spectrum (Fig. 5B). The mass spectrum of native, non-nitrated ATII shows an ion at *m/z* 524 $[M+2H]^{2+}$, but does not display any ion at *m/z* 546 or *m/z* 531 in the presence of myoglobin and DTT at 90 °C (Fig. 5C).

Due to the partial reduction of NO_2Tyr-ATII incubated with myoglobin alone at 90 °C, we applied MALDI-TOF to study effects of Hb at 70 °C (Fig. 6). In combination with DTT a complete reduction of NO_2Tyr-ATII (*m/z* 1091 for $[M+H]^+$) to NH_2Tyr-ATII (*m/z* 1061 for $[M+H]^+$) was obtained (Fig. 6A), comparable to that by dithionite which is recommended in Western blot to produce a negative control (Fig. 6B). When NO_2Tyr-ATII was incubated with Hb alone, a partial reduction was observed as indicated by the ions at *m/z* 1061 and *m/z* 1091, but interestingly also an ion at *m/z* 1075 was observed indicating loss of one oxygen atom, presumably due to reduction of the nitro group to a nitroso group in NO_2Tyr-ATII (Fig. 6C). Finally, we investigated by NanoESI–MS the effects of adding the same species as in Fig. 6, i.e. Hb and Hb + DTT to NO_2Tyr-ATII at 56 °C. Temperature and time in this experiment were chosen in accordance with the in-gel digestion protocol for proteins. In both cases, the MS spectra of NO_2Tyr-ATII showed intense ions at *m/z* 546 $[M+2H]^{2+}$, but no abundant ions at the expected *m/z* value of 531 of NH_2Tyr-ATII $[M+2H]^{2+}$ were obtained (Fig. 7).

4. Discussion

Balabanli et al. described a non-enzymatic reduction of NO_2Tyr to NH_2Tyr, which was dependent on thiols and heme-containing proteins [6]. Gel electrophoresis/Western blot uses DTT as a reducing agent, and heme-containing proteins are always present in tissue samples. The methods are executed at high temperature enhancing chemical reactions. In-gel digestion of 1D gels using trypsin also takes advantage of high temperature and thiols to digest proteins into peptides. In the present study, we provide evidence by HPLC, MS and immunoblotting (non-fixed tissue) that high temperature in combination with heme-containing proteins and DTT reduces the protein-associated nitro group to the corresponding amino group. However, we observed positive and blockable immunostaining of nitrotyrosine in zymosan-treated animals as well as controls, using immunohistochemistry (fixed tissue) in accordance with Greenacre et al. [31]. In contrast, Western blotting with monoclonal anti-nitrotyrosine IgG revealed no blockable antigen–antibody interaction. Our findings indicate potential methodological problems that should be considered when applying Western blot analysis to study protein nitration.

4.1. Reduction of the nitro group by heme and thiol groups

In the present study, we focused on the problems associated with low nitrotyrosine immunoreactivity in Western blots. Kamisaki et al. [8] observed that lung and spleen homogenates modified nitrotyrosine-containing proteins using Western blotting. Our approach was to study one nitrated peptide with and without addition of heme-containing proteins, as suggested by Balabanli et al. [6], to reveal conditions that may affect the nitro group in nitrated proteins. The chosen peptide was angiotensin II (ATII), which we and others have found to be sensitive to in vitro nitration [27,32]. We discovered that hemoglobin and myoglobin in combination with DTT reduce the 3-nitro group in nitrated angiotensin II, i.e. NO_2Tyr-ATII, to the corresponding 3-amino group containing derivative, i.e. NH_2Tyr-ATII. The structure of NH_2Tyr-ATII was identified with tandem mass spectrometry (not shown). NH_2Tyr-ATII is not recognized by anti-nitrotyrosine IgG. Our findings cannot be explained by an enzyme-dependent reduction of the nitro group. Also, it is unlikely that our results are due to proteolytic degradation [7], as was demonstrated in our gel experiment (Fig. 3A). However, the result may be explained by the heme- and thiol-dependent, non-enzymatic reduction of 3-nitrotyrosine to 3-aminotyrosine

[6]. Interestingly, hemoglobin may be a peroxynitrite scavenger of physiological relevance [33], and in a recent study, Kikugawa et al. [34] demonstrated no accumulation of nitrated proteins in circulating erythrocytes. Thus, physiological mechanisms may contribute to diminish NO_2Tyr-residues in vivo, limiting the possibility for easy detection by immunoblotting. To optimize such techniques, analytical conditions associated with increased reduction of NO_2Tyr should be avoided. Balabanli et al. [6], in attempts to avoid non-enzymatic reduction of NO_2Tyr by the addition of NO donors, found that DETA-NO or (*z*)-1-[2-(-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1 was the only one that could stop the reaction. However, we did not test this possibility in the present study.

4.2. Influence of temperature

In protocols for protein digestion (cleavage of peptide bonds) and Western blotting, the regularly used temperature is 56 °C or higher. In our MS experiments, the mixture of the nitrated peptide with DTT and myoglobin revealed a temperature-dependent conversion of the nitro group to an amino group in the peptide above 56 °C. Complete reduction was observed at higher temperatures, while no reduction of NO_2Tyr-ATII to NH_2Tyr-ATII was observed at 56 °C. 3-Nitrotyrosine reduction has previously been described by Balabanli et al. [6], who used the HPLC-electrochemical detection (ECD) of nitroaromatic compounds at 37 and 100 °C. In their experiments, complete conversion of 3-nitrotyrosine to 3-aminotyrosine was observed when incubated in PBS (pH 7.2) with DTT and hemoglobin at 100 °C, while a 15% reduction was observed at 37 °C. However, in Western blot experiments using nitrated albumin, we visually observed a non-enzymatic, temperature-dependent reduction in immunoreactivity even at 56 °C in comparison to the samples that were incubated at room temperature.

4.3. Western blotting (non-fixation) versus histochemistry (fixation)

In fixed tissues, heme-proteins have no effect on adjacent proteins. This may explain why immunohistochemistry is not influenced as much as Western blot experiments. Further, Gow et al. [7] found in non-fixed tissue and plasma stored at −80 °C, that 3-nitrotyrosine levels may decline over time. In our study, it is rather unlikely that such a decay has influenced our results, as our studies were performed in close relation with sampling.

4.4. Cross-reactivity

Cross-reactivity can be a problem for both the primary and the secondary antibodies in Western blot experiments. To reduce untoward cross-reactivity, a primary antibody produced in another species than the target was chosen. In our inflammation model, the gel was loaded with abdominal exudate proteins and the secondary antibody displayed strong immunostaining for NO_2Tyr, corresponding to a molecular weight of 52 kDa and approximately 20 kDa. Control experiments with dithionite to reduce the nitro group, and with nitrotyrosine to block the primary antibody, only slightly reduced the staining intensity, indicating a primarily unspecific reaction (cross-reaction). Western blot studies demonstrating nitrated proteins around a molecular weight of 50 kDa have used homogenate from cells or tissues not expressing IgG [15,35,36]. We were not able to see any cross-reaction of liver homogenate in our Western blot experiment. Marcondes et al. [15] found nitrated protein corresponding to 52 kDa in the kidney 6 h after zymosan treatment but not in the liver. In a 2D-gel approach, which separates proteins in two dimensions, Aulak et al. found after 18-h post-zymosan administration 31 liver proteins which were immunostained for nitrotyrosine [15,35,36]. In our study, even though we loaded the gel with a large amount of protein (∼70 μg/well), we found no immunoreactivity in the liver and no cross-reactivity to IgG using a monoclonal anti-nitrotyrosine antibody (Fig. 3D), while immunoreactions were strong over the entire protein range when the polyclonal antibody was used (Fig. 3C).

5. Conclusions

Protein nitration seems to occur even under physiological conditions, as we here demonstrate by immunohistochemistry. However, even MALDI-TOF/TOF MS analysis of albumin isolated from inflamed tissue could not detect any NO_2Tyr residues after preparation equivalent to experimental conditions for ordinary Western blot. Under similar conditions, monoclonal NO_2Tyr antibodies caused an unspecific cross reaction to the secondary antibody. Although the monoclonal antibodies could detect nitrated rat serum albumin, the immunostaining was almost abolished by high temperature under reducing conditions and, in contrast to polyclonal antibodies, it did not indicate nitration of proteins in inflamed liver tissue even at room temperature. At higher temperatures, regularly used for Western blot, in the presence of DTT or a heme-containing protein, the nitrated peptide angiotensin II (NO_2Tyr-ATII) partially retained its NO_2Tyr residue. However, when both DTT and heme-containing proteins are present, a more or less complete conversion of NO_2Tyr-ATII to NH_2Tyr-ATII took place. The latter condition would thus artificially prevent detection of protein nitration by Western blot, as anti-NO_2Tyr antibodies do not recognize NH_2Tyr. Our findings might explain a sensitivity problem with Western blot analysis and in-gel digestion. Temperatures above 56 °C, often used in Western blot, increase NO_2Tyr reduction. Reducing conditions sufficient to reduce S–S bonds but mild enough to avoid reduction of NO_2Tyr to NH_2Tyr residues seem to be a prerequisite for the application of Western blot to detect nitrated proteins.

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