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Tyrosine nitration moderates the peptidase activity of human methionyl aminopeptidase 2

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ABSTRACT

Methionyl aminopeptidase 2 (MetAP2) plays an important role in the regulation of angiogenesis. This study examined whether nitration of MetAP2 alters its enzymatic activity *in vitro*. The activity of unmodified, nitrated and oxidised MetAP2 was assessed and it was found that nitration significantly reduced its ability to cleave a chromogenic substrate. Mass spectrometry analysis identified Tyr336 as a nitrated residue in MetAP2. Structural and evolutionary analysis indicate that this is an important residue for MetAP2 activity. Combined, the results show that the activity of MetAP2 is reduced by nitration and raise the possibility that nitration of MetAP2 is a mechanism contributing to endothelial dysfunction.

1. Introduction

Methionyl aminopeptidases (MetAP) are enzymes responsible for the removal of methionine from the amino-terminus of nascent proteins [1]. These enzymes are highly specific for N-terminal methionine and also have strong preference for certain small uncharged amino acids located immediately C-terminal of the methionine [2]. Removal of the N-terminal methionine from proteins is essential for further post-translational N-terminal processing of proteins including acetylation and myristoylation [3]; as such, MetAPs play a crucial role in the post-translational processing of proteins.

In humans, two enzymes MetAP1 and MetAP2 are known to possess MetAP activity. The two proteins differ in the N-terminal and C-terminal domains. MetAP1 contains two zinc-finger motifs in the N-terminal region, while MetAP2 contains a highly charged N-terminus and an alpha-helical domain in the C-terminal part [4]. While these domains are likely to define subcellular localisation and substrate specificity of the two enzymes, roles unique to each MetAP remain poorly defined. In yeast, knockout of one of the two MetAPs results in decreased growth rates while elimination of both genes is lethal, suggesting that MetAPs have essential cellular functions [5].

MetAP2 has attracted significant scientific attention since the discovery that it is the target molecule of the potent anti-angiogenic compounds fumagillin and its analogues [6]. In cancer, human MetAP2 down-regulation has been shown to inhibit endothelial cell proliferation and angiogenesis [7]. Another study showed that the use of fumagillin inhibited tumour growth and metastasis in rats [8]. Outside of these cancer models, MetAP2 has also been found to be essential for cell proliferation in *Caenorhabditis elegans* [9]. Together, the evidence suggests that MetAP2 plays an important role in the regulation of endothelial proliferation and angiogenesis.

It has been well established that reactive nitrogen species (RNS), such as nitric oxide (NO), are key regulators of endothelial function and angiogenesis [10]. The reduction in NO level is a hallmark of endothelial dysfunction [11]. One mechanism leading to decreased NO levels is through its inactivation by reactive oxygen species (ROS) [12]. Reaction of NO and ROS leads to the formation of peroxynitrite, which readily reacts with proteins to form 3-nitrotyrosine [12]. Both increases in the level of peroxynitrite and the presence of 3-nitrotyrosine has been implicated in a range of diseases such as stroke [13], myocardial infarction [14], atherosclerosis [15] and diabetes [16], all of which are characterised by endothelial dysfunction. MetAP2 is known to play an important role in endothelial proliferation and angiogenesis, therefore, its inactivation by peroxynitrite may present a possible mechanism contributing to endothelial dysfunction and associated diseases.

In this study, the potential role of 3-nitrotyrosine formation in MetAP2 in altering its peptidase activity is investigated. MetAP2 was first nitrated *in vitro* using a heme-catalysed nitration method [17]. A chromogenic assay was then used to assess the peptidase activity of unmodified, nitrated and oxidised MetAP2. LC-MS/MS was further performed in order to identify the site of nitration on MetAP2. Subsequently, structural and evolutionary analysis of a tyrosine residue found to be nitrated was performed to support the potential of this modification to affect MetAP2 activity.

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2. Materials and methods

2.1. Nitration of MetAP2 by heme-catalysed nitration

The method of heme-catalysed nitration was adapted from Bian et al. [17]. Recombinant human MetAP2 (R&D Systems) was nitrated in the presence of hydrogen peroxide (H_2O_2), cytochrome C (Sigma Aldrich) and sodium nitrite ($NaNO_2$). Final concentrations of 0.75 μM MetAP2 in buffer (50 mM HEPES, 0.1 mM $CoCl_2$, 100 mM NaCl, pH 7.5) were prepared. Cytochrome C was added to a final concentration of 7.9 μM and $NaNO_2$ to 1 mM. A stock of 30% hydrogen peroxide solution (9.8 M) was diluted and added to sample aliquots without delay. Samples were incubated at 37 °C in a thermomixer (Eppendorf) with gentle agitation for 15 min. Nitration reactions were halted with 3.3 mM methionine and samples were chilled on ice before further sample treatment.

To isolate the effects of hydrogen peroxide oxidation on the activity of MetAP2, the heme mediated nitration reaction was also performed in the absence of $NaNO_2$, in matching equimolar H_2O_2 concentrations. Multiple aliquots of MetAP2 were diluted with cytochrome C as described and were treated with 1.5, 0.15 and 0.015 mM H_2O_2 in the absence of sodium nitrite. Concurrently, matched aliquots of MetAP2 were nitrated as per protocol described for the different levels of H_2O_2 and 1 mM $NaNO_2$.

2.2. Quantitative MetAP2 activity assay by N-terminal cleavage of substrate 7-amino-4-methyl-courmarin

The activity of MetAP2 was measured using fluorescent peptide substrate Met-Gly-Pro-AMC (R&D systems) as described by Garrabrant et al. [18]. Briefly, the reaction (100 μl final) was initiated by adding 0.5 μg of MetAP2 to reaction buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 mM $CoCl_2$, 1 mg/ml PEG6000, 0.0025 activity units of porcine dipeptidyl peptidase (Sigma–Aldrich), and 0.5 mM Met-Gly-Pro-AMC substrate. The assay was prepared in black flat-bottomed 96-well microtiter plates and fluorescence was measured every 20 s over a period of 100 min at 25 °C using a Fluoroskan Ascent microplate fluorometer (Thermo Scientific), with excitation and emission wavelengths set at 355 and 460 nm respectively. Specific activity was calculated from a calibration standard of 7-amino-4-methyl-courmarin.

2.3. Identification and confirmation of protein 3-nitro-tyrosine by western blot analysis

Affirmation of protein-3-nitro-tyrosine formation was conducted by western blotting using a monoclonal anti-nitro-tyrosine antibody (Sigma Aldrich, N5538). Aliquots of sample protein were diluted to 0.5 μg per 10 μL aliquot and equal volumes of a 2 × LDS sample loading buffer with 20 mM DTT were combined and boiled for 5–10 min. Samples were then resolved on a SDS–PAGE gel. On completion of electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane and western-blot was performed. In some experiments PVDF membrane was stripped of primary antibodies and re-probed with anti-MetAP2 antibody (Abcam, ab124953).

2.4. Mass spectrometry identification of protein-3-nitrotyrosine

Nitrated MetAP2 protein was resolved on a SDS–PAGE gel and stained with colloidal coomassie. The protein band was excised from the gel, destained, dried and incubated with 100 mM DTT in 25 mM $(NH_4)HCO_3$ for 1 h at 25 °C. The gel slice was washed with $(NH_4)HCO_3$, dried and incubated with 55 mM iodoacetamide in 25 mM $(NH_4)HCO_3$. The slices were washed and dried before digestion of the nitrated protein with 20 ng μL^{-1} trypsin in 25 mM $(NH_4)HCO_3$ overnight at 30 °C.

Peptides were eluted from the slices with 5% formic acid and 50% acetonitrile for 30 min and separated by nano-LC on an Ultimate 3000 HPLC (Dionex) using a fritless nano column (75 μ × ~10 cm) containing C18 media (5 μ, 200 Å Magic, Michrom). Peptides were eluted using a linear gradient of H_2O:CH_3CN (98:2, 0.1% formic acid) to H_2O:CH_3CN (64:36, 0.1% formic acid) at 250 nL min^{-1} over 60 min. Positive ions were generated by electrospray and analysed in a LTQ FT Ultra (Thermo Electron) mass spectrometer operated in data catalysed MS/MS acquisition mode.

Mass spectral data was searched using Mascot (V2.3, Matrix Science) against the Human Uniprot database (2013_02), common contaminant proteins and a reverse decoy database. Search parameters included were: precursor tolerance 6 ppm and product ion tolerances ±0.8 Da. Cys-carboxyamidomethyl as a fixed modification and Tyr-NO_2, Met-O, Trp-O, Acetyl (Protein N-term) and Pyro-Glu/Gln as variable modifications with full tryptic or chymotryptic cleavage of up to 3 missed cleavages. Significance threshold was set at $p < 0.01$ which resulted in a false discovery rate of 2% for the tryptic digest sample and 5% for the chymotryptic sample.

2.5. Three-dimensional structural analysis of MetAP2

The X-ray crystal structure of full length MetAP2 with the highest resolution, 1B6A, was used [19]. The structure was visualised and analysed using Accelrys Discovery Studio (v3.5). A 5 Å radius was used to identify neighbouring amino acid side-chains to the Tyr336 aromatic functional group. Annotations of cobalt and substrate binding amino acids were obtained from Uniprot/Swiss-prot which was curated from crystal structures of MetAP2 [20].

2.6. Sequence conservation analysis of MetAP2

MetAP2 protein sequences were obtained from Uniprot/Swiss-prot. The following sequences were analysed: P50579 (*Homo sapiens*; HUMAN), O08663 (*Mus musculus*; MOUSE), P38062 (*Rattus norvegicus*; RAT), Q3ZC89 (*Bos taurus*; BOVINE), F7DQE9 (*Xenopus tropicalis*; FROG), H9K8M7 (*Apis mellifera*; HONEYBEE), Q55C21 (*Dictyostelium discoideum*; SILM MOULD), P38174 (*Saccharomyces cerevisiae*; YEAST), Q8SR45 (*Encephalitozoon cuniculi*, MICROSPORIDIA) and Q6RC05 (*Trypanosoma brucei*; PROTIST). Multiple sequence alignment of all sequences was performed with ClustalW [21] using default parameters.

3. Results and discussion

3.1. Nitration of MetAP2 by heme-catalysed nitration alters protein activity

Heme-catalysed nitration of MetAP2 was performed by treating the protein in the presence of $NaNO_2$, H_2O_2, and a heme source in the form of cytochrome C. Varying concentrations of H_2O_2 was used ranging from 0.015 to 1.5 mM. There was a clear increase in MetAP2 nitration with increasing concentrations of H_2O_2 as determined by anti-3-nitro-tyrosine western blot (Fig. 1A). This was accompanied by a degree of protein degradation/aggregation as evidenced by additional bands in the anti-MetAP2 western blot and protein silver stained gel. However, based on the silver stained gel, although there is protein loss compared with the untreated control, there was no substantial further degradation of MetAP2 with increasing H_2O_2 treatment.

In order to test whether the activity of MetAP2 is affected by heme-catalysed nitration, MetAP2 activity was again assayed based on its ability to cleave a chromogenic substrate. It is evident

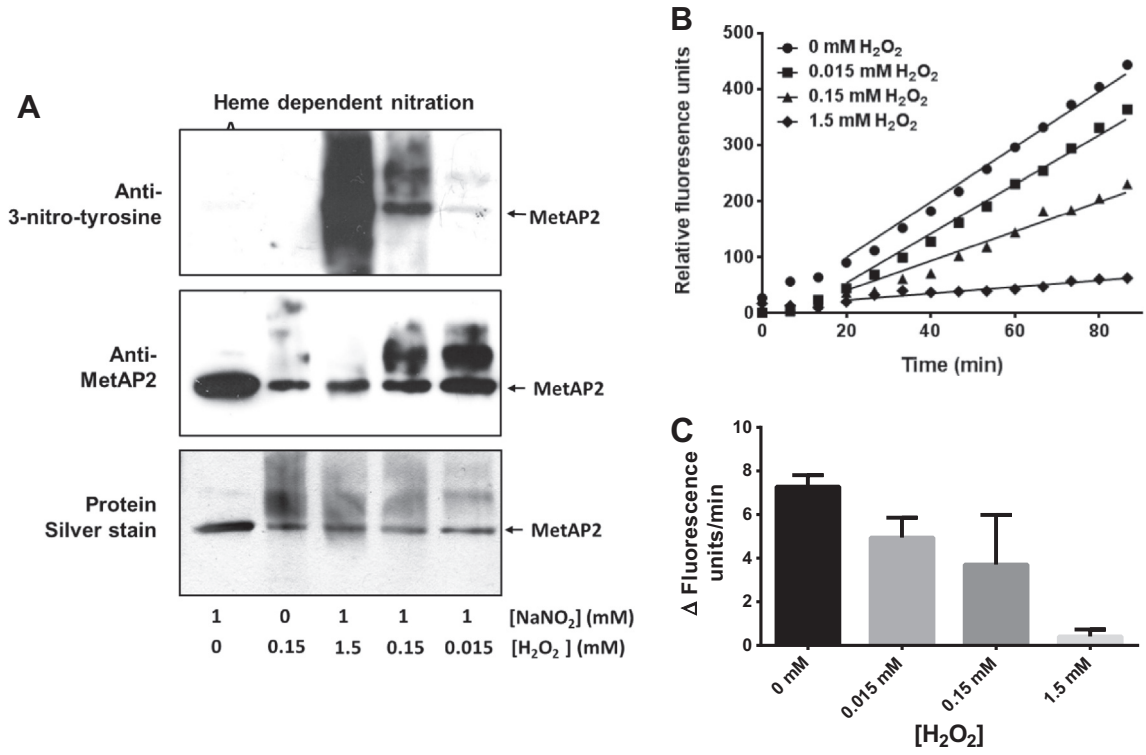

Fig. 1. Heme-catalysed nitration of MetAP2. (A) Anti-3-nitrotyrosine and silver stained protein gel of MetAP2 treated with 1 mM $NaNO_2$ and increasing concentrations of H_2O_2. (B). Chromogenic assay of MetAP2 activity with 1 mM $NaNO_2$ and increasing concentrations of H_2O_2. (C) Change in rate of chromogenic substrate cleavage averaged over three independent experiments. Statistical significance of the rate of change over all conditions was evaluated using ANOVA ($p < 0.01$).

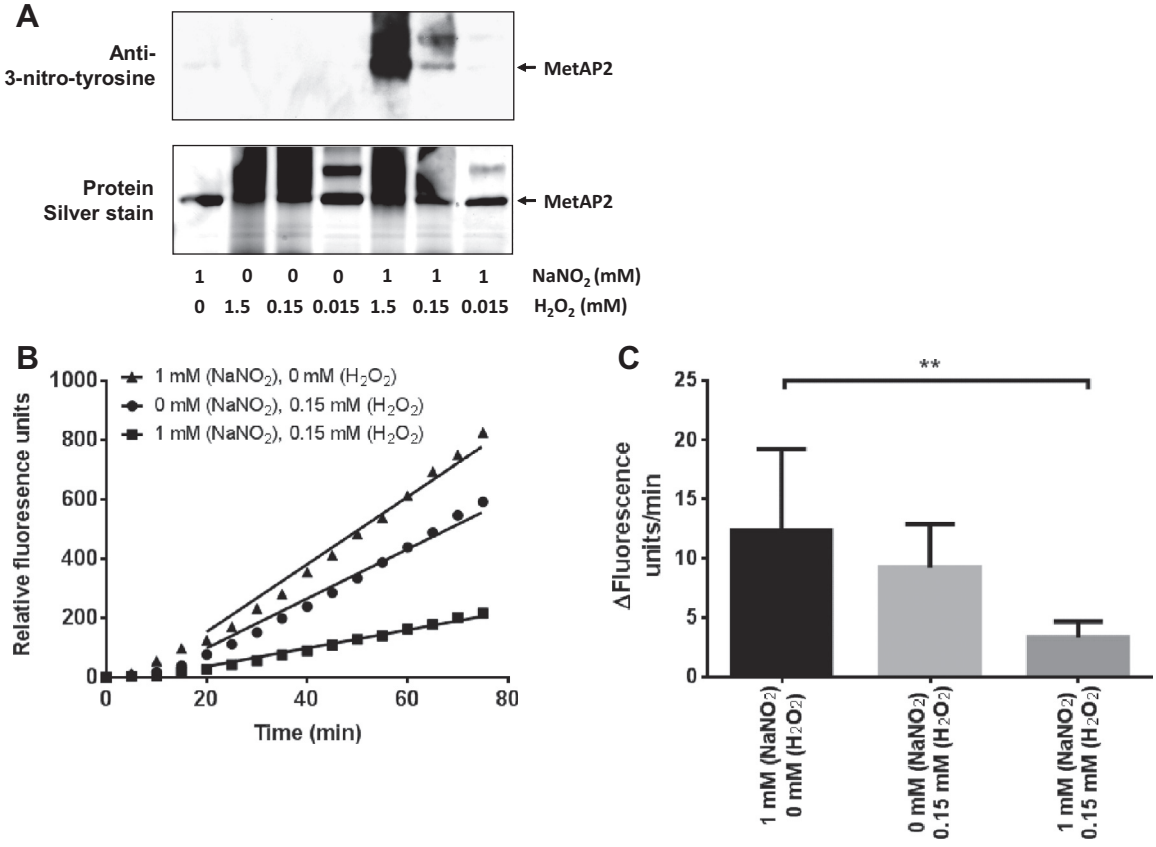

Fig. 2. Comparison of nitration and oxidation of MetAP2. (A) Anti-3-nitrotyrosine blot and silver stained gel of nitrated and oxidised MetAP2. (B) Chromogenic assay of MetAP2 activity with or without 1 mM $NaNO_2$ and 0.15 mM H_2O_2 along with a control where no H_2O_2 was present. (C) Change in rate of chromogenic substrate cleavage averaged over three independent experiments. Statistical significance was evaluated using a paired *t*-test ($^{**}p < 0.01$).

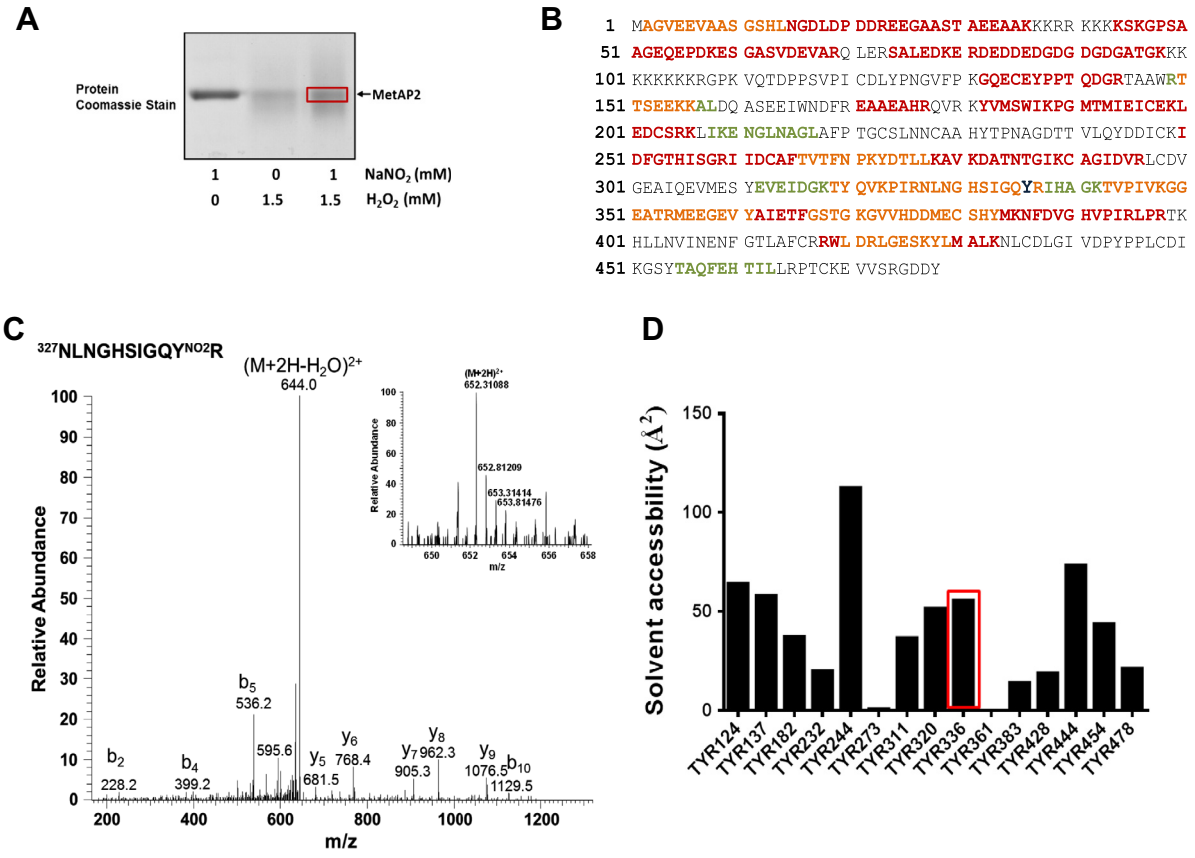

Fig. 3. Mass spectrometry analysis of nitrated MetAP2. (A) Coomassie stained gel with excised nitrated MetAP2 band boxed in red. (B) MS peptide coverage of MetAP2. Residues detected in tryptic peptides only are in red, residues detected in chymotryptic peptides only are in green and resides detected using both enzymes are in orange. The nitrated tyrosine (Tyr336) is highlighted in blue and larger font. (C) The MS/MS spectrum of the nitrated tryptic peptide containing nitrated Tyr336. The MS1 accurate mass spectrum is shown in the inset. (D) Solvent accessibility of tyrosine residues in MetAP2. The solvent accessibility was determined from 1B6A using GetArea [22]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

that there was a decrease in the rate of peptide cleavage with increasing concentrations of H_2O_2 treatment (Fig. 1B). When the rate of cleavage was expressed as the change in fluorescence over time and averaged over three independent experiments, the rates of cleavage were found to be significantly different across H_2O_2 dosages (Fig. 1C, $p < 0.01$, ANOVA).

While the decrease in activity at 0.015 mM H_2O_2 compared with the untreated sample may be attributed to the degradation of MetAP2 protein, since further degradation is not apparent with further increases in H_2O_2, the further decreases in activity are likely due to the nitration of MetAP2.

3.2. Loss of MetAP2 activity is largely due to tyrosine nitration rather than protein oxidation

An advantage of the heme-catalysed nitration method is that the procedure allows nitration to be directly compared with oxidation under the same conditions. By excluding nitrite (NO_2^{2-}), from the reaction, only oxidation can occur to amino acid side chains. In order to determine whether nitration or oxidation contributes to the reduction of MetAP2 activity, MetAP2 was treated with cytochrome C at varying concentrations of H_2O_2 with and without $NaNO_2$. From the anti-nitrotyrosine western blot of MetAP2, it is evident that nitration can only be detected where $NaNO_2$ was present. In terms of total MetAP2 protein, when H_2O_2 was present, degradation occurred with or without $NaNO_2$, suggesting that H_2O_2 is the main driver of protein degradation (Fig. 2A).

The activity of nitrated and oxidised MetAP2 was compared in the 0.15 mM H_2O_2 treatment with and without 1 mM $NaNO_2$ against a control where only 1 mM $NaNO_2$ was present. The activity of oxidised MetAP2 was lower than that of the control, which can be expected as the protein gel showed significant protein degradation/aggregation (Fig. 2B). Interestingly, in the present of $NaNO_2$ the activity of MetAP2 dropped further without any significant increase in protein degradation/aggregation. Comparison of the rate of change in florescence over time across three independent experiments showed that the activity of nitrated MetAP2 was significantly lower than the control (Fig. 2C, $p = 0.0066$, paired t-test). This result suggests that nitration rather than oxidation is the main driver for the reduction of MetAP2 peptidase activity.

3.3. Mass spectrometry analysis identifies nitrated residue in MetAP2

In order to identify 3-nitrotyrosines that may be responsible for the reduction in MetAP2 enzyme activity, LC-MS/MS was used. Results from the previous experiments showed that nitrotyrosine could be detected significantly only with H_2O_2 concentration of>0.15 mM (Fig. 1A). To ensure that sufficient nitrotyrosine would be present to be detected by MS, MetAP2 was treated with 1.5 mM H_2O_2 and 1 mM $NaNO_2$. The nitrated protein was resolved on SDS–PAGE, coomassie stained and the nitrated MetAP2 band was excised and digested (Fig. 3A). To ensure maximal protein coverage, the experiment was performed twice using trypsin and chymotrypsin once each for proteolysis prior to LC-MS/MS analysis. Combining the proteolytic MetAP2 peptides from the two

experiments identified by Mascot provided 64.5% coverage of the protein (Fig. 3B). A single nitrotyrosine was identified at Tyr336. The tandem mass spectra was analysed and annotated manually to confirm the identification of the nitrated peptide by Mascot (Fig. 3C). The full mass spectrometry results can be found in the Supplementary tables.

Although this study identified a single tyrosine residue, Tyr336, to be nitrated in MetAP2, examination of the MetAP2 protein sequence shows that residues 218–249 are challenging to sequence using conventional LC-MS/MS methods since the region lacks trypsin cleavage sites and even the use of alternative enzymes such as chymotrypsin or GluC do not theoretically yield ideal peptides for MS analysis. As such, it is possible that Tyr232 may be nitrated, but unidentifiable in this experiment.

Tyr336 is a relatively solvent exposed residue as determined using the GetArea algorithm [22], but is not the most exposed tyrosine in MetAP2 (Fig. 3D). This suggests that local sequence, secondary structure and adjacent amino acids may play a role in dictating nitration. This is in line with previous studies which have found that the specificity of tyrosine nitration is dependent on a number of factors. In fact, analysis of the amino acids adjacent to Tyr336 shows that there is a glutamine in the −1 position as well as a positively charged arginine at+1, conforming to the previously reported sequence context of 3-nitrotyrosines [23].

3.4. Tyrosine 336 is located adjacent to the peptide binding pocket of MetAP2 and is highly conserved

In order to investigate how nitration of Tyr336 in MetAP2 may alter the activity of the protein, X-ray crystal structure of MetAP2 was assessed. Tyr336 is located at a loop between two beta strands that is part of the peptide binding pocket of MetAP2 (Fig. 4A). Importantly, the two connecting beta strands contain key histidine residues important in cobalt (His331) and substrate peptide binding (His339) (Fig. 4B) [20]. The tyrosine is also in close vicinity (within 5 Å) of the loops of other beta strands that form part of the binding pocket (Fig. 4C). Therefore nitrated Tyr336 can also potentially alter the conformation of other parts of the substrate binding pocket. Finally, it has also been shown that the nitration of tyrosine alters the pKa of the side chain from 10.1 to 7.1 [24]. The electrostatic interaction between nitrotyrosine and adjacent residues, therefore, may also be affected. Together, this suggests that the nitration of Tyr336 can be expected to alter the structure of the MetAP2 substrate binding pocket and consequently alter MetAP2 enzymatic activity.

To further assess the evolutionary importance of Tyr336, the conservation of this residue was analysed. MetAP2 protein sequences across a representative selection of eukaryotes including vertebrates, insects, fungi and protists were aligned to assess conservation (see Supplementary figure for full alignment). Based on the multiple sequence alignment, Tyr336 was conserved across all species except in the microsporidian parasite, *E. cuniculi*, which had a generally divergent MetAP2 protein sequence [25]. Nevertheless, phenylalanine, which like tyrosine also bears an aromatic side-chain is still present at the same position in *E. cuniculi* suggesting that the position is very important for maintenance of MetAP2 function. Together, the position of Tyr336 in the structure and its conservation across eukaryotes suggests that the residue is crucial for MetAP2 activity and therefore, nitration of Tyr336 is expected to affect MetAP2 peptidase activity. Given the role of MetAP2 in endothelial proliferation and angiogenesis, and the implication of peroxynitrite in endothelial dysfunction, the results of this study suggests that nitration of MetAP2 by peroxynitrite may be a possible mechanism contributing to endothelial dysfunction.

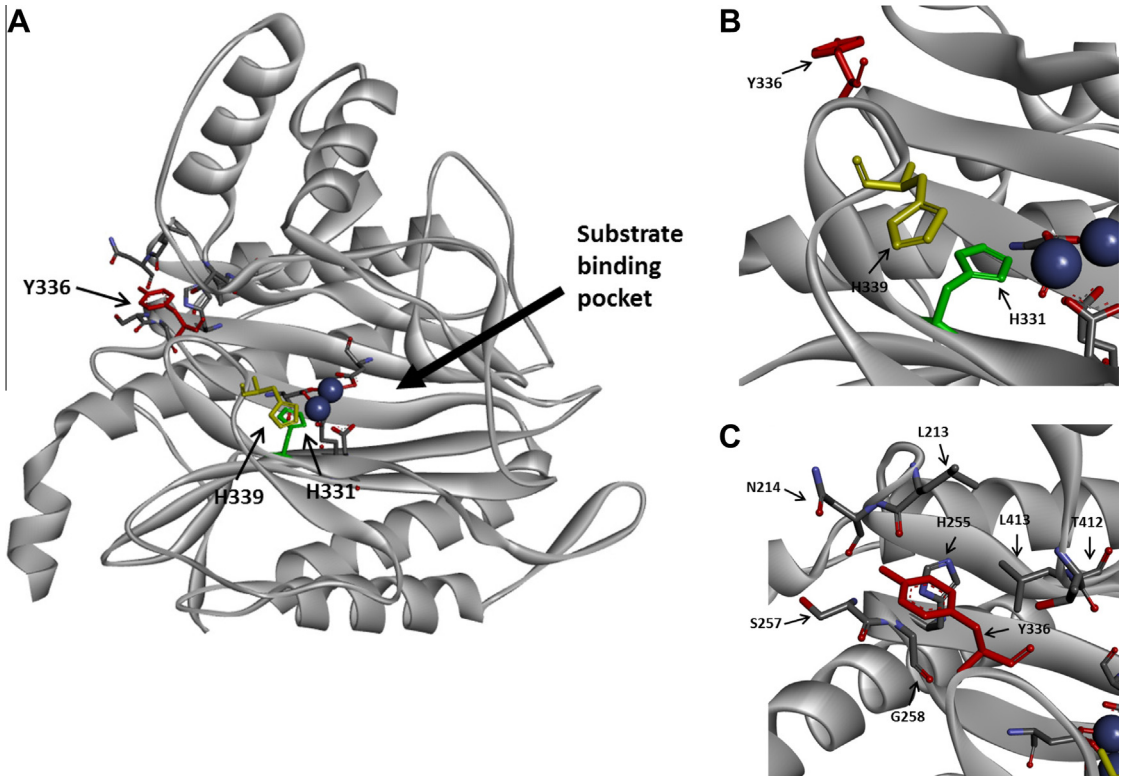

Fig. 4. Structural analysis of MetAP2. (A) X-ray crystal structure of MetAP2 (PDB: 1B6A). Tyr336 is highlighted in red, His331 in green and His339 in yellow. Residues within 5 Å of Tyr336 are shown as are residues involved in cobalt binding. (B). Zoomed-in region showing that His331 and His339 are on strands immediate adjacent to Tyr336. (C) Zoomed-in region showing residues within 5 Å of Tyr336. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.035.

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