Proteomics. Author manuscript; available in PMC 2009 November 16.

Published in final edited form as:
Proteomics. 2007 September ; 7(17): 3066–3084. doi:10.1002/pmic.200700254.

Human Spermatozoa Contain Multiple Targets for Protein S-Nitrosylation: An Alternative Mechanism of the Modulation of Sperm Function by Nitric Oxide?

Linda Lefièvre[1], **Yongjian Chen**[2], **Sarah J Conner**[1,3], **Joanna L Scott**[1], **Steve J Publicover**[4], **W Christopher L Ford**[1], and **Christopher LR Barratt**[1,†]

[1]Reproductive Biology and Genetics Group, Division of Reproductive and Child Health, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

[2]The Center of Reproductive Medicine, Peking University Third Hospital, Beijing, China, 100083

[3]Centre for Human Reproductive Science, Birmingham Women's Hospital, Metchley Park Road, Edgbaston, Birmingham, B15 2TG, UK

[4]School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK

Abstract

Nitric oxide (NO) enhances human sperm motility and capacitation associated with increased protein phosphorylation. NO activates soluble guanylyl cyclase, but can also modify protein function covalently via S-nitrosylation of cysteine. Remarkably, this mechanism remains unexplored in sperm although they depend on post-translational protein modification to achieve changes in function required for fertilisation. Our objective was to identify targets for S-nitrosylation in human sperm. Spermatozoa were incubated with NO donors and S-nitrosylated proteins were identified using the biotin switch assay and a proteomic approach using tandem mass spectrometry. 240 S-nitrosylated proteins were detected in sperm incubated with S-nitrosoglutathione. Minimal levels were observed in glutathione or untreated samples. Proteins identified consistently based on multiple peptides included established targets for S-nitrosylation in other cells e.g. tubulin,, glutathione-S-transferase and heat shock proteins but also novel targets including A-kinase anchoring protein (AKAP) types 3 and 4, voltage-dependent anion-selective channel protein 3 and semenogelin 1 and 2. *In situ* localisation revealed S-nitrosylated targets on the post-acrosomal region of the head and throughout the flagellum. Potential targets for S-nitrosylation in human sperm include physiologically significant proteins not previously reported in other cells. Their identification will provide novel insight into the mechanism of action of NO in spermatozoa.

Keywords

Human; Nitric Oxide; Signalling; S-Nitrosylation; Spermatozoa

1. Introduction

The mature mammalian spermatozoon is a ‘minimalist’ cell, specialised for its sole purpose of delivering the haploid nucleus and activating factors to the female gamete. To this end

[†]To whom correspondence should be addressed: c.l.barratt@bham.ac.uk, Room WX 1.30, West Wing, Medical School, Reproductive Biology and Genetics Group, Division of Reproductive and Child Health, University of Birmingham, Birmingham B15 2TT Tel: +44 121 414 7628.

Europe PMC Funders Author Manuscripts

'surplus' cytoplasm, including all endoplasmic reticulum, is jettisoned during spermiogenesis (the final stage of differentiation). The differentiated sperm thus lacks functional apparatus for DNA transcription or protein expression. However, fully-differentiated spermatozoa must undergo profound functional changes, both during maturation in the epididymis and upon interaction with the female reproductive tract [1]. These events, which are vital for fertilisation to occur, depend on post-translational modification of proteins (e.g. tyrosine phosphorylation) [2] in the mature cells.

Human spermatozoa undergo extensive redox regulated signalling [3, 4] and a plethora of studies have documented a significant positive effect on sperm function upon the addition of exogenous nitric oxide (NO) [5-7]. For example, NO is involved in sperm motility [8], capacitation (a maturational process spermatozoa must undergo before they can fertilize oocytes) [9], acrosome reaction [9], and enhancement of sperm-zona pellucida binding ability [10]. Human spermatozoa have both endothelial and neuronal forms of nitric oxide synthase (endothelial, eNOS and neuronal, nNOS) [8, 11-15]. However, the significance of these enzymes in signalling in mature sperm is debatable (see discussion). Perhaps more significantly, spermatozoa are exposed to a significant array of highly effective NO producing cells in the human female tract (concentration from 5nM to 4μM; Marcondes *et al.* [16] with which they have intimate and prolonged [hours] contact. The exact site(s) of production of NO in the female tract and the temporal sequence of events remains to be investigated but evidence from our laboratory suggests that cumulus cells surrounding the oocyte produce easily detectable amounts of NO [17] (unpublished data), implying that NO from this source greatly exceeds endogenous production by sperm.

The question is, 'how does NO affect human sperm function?' Until recently, it was generally accepted that NO effects were mediated principally through cGMP-related mechanisms. Stimulation of soluble guanylyl cyclase is a well-characterised mode of action for NO and the importance of cGMP signalling in the sperm of marine invertebrates is also well established [18]. However, the concentration of cGMP and the activities of cGMP-specific phosphodiesterase (type 5) and protein kinase G are low or undetectable in human spermatozoa [12, 19]. To date, the primary role of cGMP in human sperm (as shown by use of specific kinase inhibitors and addition of dibutyryl cGMP) is induction of the acrosome reaction [18, 20]. Studies on capacitation have shown that NO also modifies activities of other kinases. Application of exogenous NO is associated with an increased cAMP levels (leading to tyrosine phosphorylation) [21] and NO is also involved in activation of a protein extra cellular signal regulated kinases (ERKs) [22, 23]. However, the exact role and site of action of NO remains speculative and the molecular mechanisms incompletely understood [23].

An alternative, cGMP-independent and potentially crucial action of NO is the covalent modification of proteins via S-nitrosylation - the formation of S-NO bond by cysteine thiol nitrosylation to form a nitrosothiol [24, 25]. S-nitrosylation is a selective, temporal and spatially regulated post-translational protein modification which regulates physiological cellular signalling, analogous to phosphorylation and acetylation [25, 26]. Over 120 proteins, from both animal and plant cells have been shown to undergo S-nitrosylation [25, 27-30] and an increasing number of proteins are shown to be functionally regulated by S-nitrosylation e.g. estrogen receptor [31] connexin 43 hemichannels [32], dynamin [29]. Remarkably, this mechanism of action remains unexplored in sperm even though NO is a well know modulator of sperm function and these remarkable cells are exclusively dependent on post-translational protein modification to effect physiological changes in function necessary for fertilisation.

The objective of our study was to perform a systematic assessment of potential targets for protein S nitrosylation in human spermatozoa using the biotin switch assay. We identified 240 S-nitrosylated proteins. This global assessment allows critical insight into the role of NO in sperm physiology, identifies potential novel mechanisms of action for S-nitrosylation and opens up a new signalling regimen in the spermatozoon which will act as a platform for further detailed studies.

2. Materials and Methods

2.1 Materials

Spermine NONOate was purchased from Molecular Probes (distributed by Invitrogen Ltd, Paisley, UK), S-Nitrosoglutathione from Merck Biosciences Ltd. (Beeston, Nottingham, UK), protease inhibitor cocktail tablets from Roche Diagnostics Ltd. (Lewes, East Sussex, UK) and EZ-Link Biotin-N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (EZ-Link Biotin-HPDP) from Perbio Science UK Ltd, (Cramlington, Northumberland, UK). Nitrocellulose membrane was supplied by GE Healthcare UK Ltd. (St.Giles, Bucks, UK), IgG Fraction Monoclonal Mouse Anti-Biotin and FITC-donkey anti-rabbit IgG conjugated to fluorescein isothiocyanate by Jackson Immunoresearch Laboratories (Stratech Scientific, Soham, Cambrigshire, UK) and Lumi-GLO, an enhanced chemiluminescence kit, from Insight Biotechnology Ltd. (Wembley, Middlesex, UK). The Silver Stain Plus was purchased from Bio-Rad Laboratories Ltd. (Hempstead, Hertfordshire, UK). Texas Red®-2-sulfonamidoethyl methanethiosulfonate (TEXAS RED®-MTSEA) was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada) and the fluorescence mounting medium from Dako Cytomation Ltd. (Ely, Cambridgeshire, UK). All other chemicals were purchased from Sigma (Poole, Dorset, UK).

2.2 Donors

Semen samples were provided by research donors who attended the Assisted Conception Unit, Birmingham Women's Hospital (Human Fertilisation and Embryology Authority Centre #0119). Donors gave informed consent under COREC ethical approval (South Birmingham REC #2003/239). All procedures were in accordance with HFEA 6th Code of Practice. Donors had normal semen characteristics as assessed by the World Health Organisation guidelines [33].

2.3 Sperm Preparation

Semen samples were obtained after 2–3 days of sexual abstinence. After liquefaction, 1 ml of semen was layered over 1 ml fractions of 45 and 90% Percoll-made isotonic with M medium (1X: 137 mM NaCl, 2.5 mM KCl, 20 mM HEPES, 10 mM glucose). Samples were centrifuged at 2000 g for 20 min. Sperm concentration was determined using an improved Neubauer haemocytometer and at least 400 cells were scored [34]. Percoll-washed spermatozoa were further washed with Phosphate Buffered Saline (PBS) to remove Percoll. Spermatozoa were then diluted and incubated in PBS.

2.4 Biotin-Switch Assay

S-nitrosylated proteins in human spermatozoa were detected using the biotin switch assay as described previously [26] with minor modifications. Briefly intact spermatozoa (50×10^6 cells/ml) were incubated in the presence of nitric oxide donors of different classes, S-nitroso-glutathione (GSNO), L-nitroso-cysteine (CSNO) and N–(2–aminoethyl)–N–(2–hydroxy–nitrosohydrazino)–1, 2–ethylenediamine (spermine NONOate), control compounds (glutathione [GSH] and exhausted CSNO) or no treatment for 1 hr at 37°C. Following incubation, spermatozoa were centrifuged at 2000g for 5 min and supernatant removed,

spermatozoa were then resuspended in lysis buffer (250 mM Hepes pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% Triton, 2.5% SDS) and protease inhibitors added to the cells. Samples were incubated for 5 min at RT and then centrifuged at 2000g for 5 min. Protein concentration was assessed and adjusted to less than 0.5mg/ml. Proteins were then precipitated using 4 volumes of ice-cold acetone for 20 min at −20°C, centrifuged at 2000g for 5min at 4°C, washed twice with 70% acetone and dried out. Precipitate was resuspended in HEN medium (250 mM Hepes pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) containing 2.5% SDS. Free thiols were blocked with a rapidly thiol-reactive agent such as methyl methanethiosulfonate (MMTS; 20 mM) for 30 min at 50°C. After blocking, extracts were precipitated with acetone as described above to remove MMTS and resuspended in HEN medium containing 1% SDS. Finally, 1mM biotin-HPDP and 1mM ascorbate were added and incubated for 1hr at 25°C to achieve biotinylation. Samples were further acetone precipitated as described above. Pellets were resuspended in 1X SDS-PAGE loading buffer (final concentration: 2% sodium dodecyl sulphate (SDS), 10% glycerol, 62.5 mM Tris–HCl, pH 6.8) without reducing agents and the samples were resolved by SDS-PAGE (10%) and transferred for immunoblotting. All steps preceding SDS-PAGE were carried out in the dark. This experiment was repeated six times. Each time either a single or a pool of two ejaculates was used. Different donors were used on each occasion.

2.5 Immunodetection of Proteins by Western Blotting

Proteins were separated by electrophoresis on SDS–PAGE (10%) gels and electrotransferred onto nitrocellulose membrane. Nonspecific binding sites on membranes were blocked with 5% (w/v) dry skimmed milk in Tris-buffered saline (0.9% NaCl, 20 mM Tris–HCl, pH 7.8) supplemented with 0.1% Tween-20 (TTBS) for the detection of biotinylated proteins. The membranes were incubated for 1 h at room temperature with the anti-biotin antibody (1/10 000). The membranes were then extensively washed with TTBS, incubated with corresponding secondary antibody conjugated with horseradish peroxidase for 1 h and again extensively washed with TTBS. Positive immunoreactive bands were detected by chemiluminescence using Lumi-GLO, according to the manufacturer's instructions. Silver staining of the proteins transferred on the nitrocellulose membrane was performed after the detection to confirm equal protein loading for all samples [35].

2.6 *In Situ* S-Nitrosoprotein Detection

Localisation of S-nitrosoproteins *in situ* was done using a method adapted from Yang and Loscalzo [36]. This method depends on blocking thiols with a thiol-reactive agent such as MMTS, followed by reducing the S-nitrosothiols with ascorbate, after which the thiols generated by ascorbate reduction are labelled with a fluorescent derivative of methanethiosulfonate (MTSEA). Briefly, spermatozoa were incubated with or without GSH or GSNO for 1 hr at 37°C. Cells were then fixed on slides using 4% formaldehyde at room temperature for 6 min and slides were washed 3 times with HEN containing 0.1% Triton X-100 for 5 min to permeabilise the cells. Thiol groups were then blocked with 20mM MMTS in HEN at 50°C for 30 min. The cells were then washed four times with HEN, after which they were incubated with 1 mM ascorbate and 0.4 μM MTSEA-Texas red in HEN at room temperature for 1 h. Excess dye was removed by washing the cells repeatedly with HEN. Stained cells were then treated with Prolonged Antifade Mounting Medium. All images were taken using an imaging system running Openlab (v. 3.1.7, Improvision Ltd, Coventry UK) with a Hammamatsu Orca Camera attached to a Nikon TE2000 microscope. Fluorescence and Hoffman Modulation Contrast images were take with LWD40X/0.55 MC3 HMC and fluorescence with 100X/1.30 oil Plan Fluor DIC H lens.

2.7 Purification of S-Nitrosylated Proteins

Biotin-labelled proteins were purified according to Foster and Stamler [26] with modifications. Briefly, after biotinylation, proteins were submitted to two round of acetone precipitation and pellets were resuspended in 1/10 diluted HEN/10 media (10X dilution of HEN) containing 1% SDS per mg of proteins. Three volumes of neutralisation buffer (20 mM Hepes pH 7.7, 100 mM NaCl, 1mM EDTA and 0.5% Triton X-100) were added. The mixture was incubated overnight at 4°C with 50μl of streptavidin agarose (50% slurry) per mg of initial proteins. Beads were previously washed twice with Neutralisation buffer and centrifuged at 200g for 10 sec. Once the incubation terminated, the beads were washed 10 times with 500μl of Wash buffer (neutralisation buffer containing 600mM NaCl). Proteins were eluted with 1X SDS sample buffer containing 90 mM of DTT. Samples were boiled for 5 min at 100°C and centrifuged at 14 000 g for 5 min. The eluate was collected and proteins were separated by SDS-PAGE (10%) and visualised by silver staining kit as described in the manufacturer's instructions. Major protein bands (between 10 and 31 bands) were excised and proteomic analyses were performed as described below. The data represents 5 independent experiments, for each of these experiments 4-6 ejaculates (from different donors) were pooled.

2.8 Mass Spectrometry/In-Gel Protein Digestion

Bands from stained 1D SDS gels were cut out and treated with trypsin as previously described [37]. Tryptic peptides obtained from individual bands were subjected to tandem mass spectrometry (MS/MS) on a nanoESI Q-Tof mass spectrometer following separation of peptides using capillary liquid chromatography with a 15 cm C18 PepMap column as previously described [37, 38]. Following MS/MS the raw data were processed using MassLynx 3.5 (Micromass). The resulting tryptic peptide de novo sequences data were then compared with the MSDB non-identical protein sequence and SwissProt curated protein databases using MASCOT software (Matrix Science Ltd, London, UK). Following matching of a peptide to a protein, the quality of the raw MS/MS data was validated (this required the presence of good quality y-ion data). Finally, each of the peptides was used to BLAST search to confirm that the protein identified by MASCOT was the only relevant match in the non-redundant protein database for a particular peptide sequence [39].

2.9 Protein distribution and function

The Gene Ontology database (www.geneontology.org/) was searched for each of the proteins identified as being S-nitrosylated in human sperm as listed in Table 1. For each protein the cellular compartment and function was noted. Gene Ontology entries were not available (March 2007) for all the proteins we identified and these have been reported as ‘No data’.

We interrogated available literature for previous reports of S-nitrosylation for each protein we identified and we believe these to be correct at the time of writing (March 2007).

3. Results and Discussion

3.1 Detection of S-Nitrosylated Proteins in Human Spermatozoa

To detect S-nitrosylated proteins, we used the biotin-switch assay developed by Jaffrey *et al.* [40] which is a sensitive and selective methods that is based on the labelling of S-nitrosylated cysteine on targeted proteins with a biotin moiety. This assay is based on the ability of ascorbate selectively to reduce S-nitrosothiols without reducing disulfide bonds. The method involves three sequential steps: blockage of the cellular free thiols by incubation with a thiol-specific methylthiolating agent (MMTS), specific reduction of S-nitrosothiols to

thiols by ascorbate, and labelling of the nascent thiol with biotin. Detection of biotinylated proteins was performed by immunoblotting with an anti-biotin antibody [40].

After sperm were incubated with GSNO (Figure 1) or with other NO donors such as S-nitrosocysteine [CSNO] or spermine NONOate (data not shown), over 30 protein bands comprising a wide range of molecular weights were detected with the biotin switch assay (Figure 1). By contrast, in untreated samples or samples treated with GSH, only a few weak protein bands could be seen, confirming that the detected bands observed in the GSNO-treated samples can be attributed to NO (Figure 1). The very low levels of staining in control preparations (no treatment, GSH or exhausted CSNO) confirm that the vast majority of thiols were successfully blocked by MMTS and were unable to react with biotin-HPDP. Moreover, analyses of GSNO-treated extracts that underwent the biotin switch method without a blocking step (no MMTS) resulted in high levels of non-specific unspecific biotinylation (Figure 1A). The low levels of S-nitrosylated proteins observed in untreated, GSH and exhausted CSNO-treated cells may represent endogenous S-nitrosylated proteins.

No reactivity was detected in GSNO-treated spermatozoa in which biotin-HPDP was omitted (Figure 1A). Furthermore, no protein bands could be detected in GSNO-treated samples that underwent the biotin switch procedure followed by DTT treatment (Figure 1A). This data demonstrate that the anti-biotin antibody shows no non-specific cross reaction with unlabeled proteins and that no *in vivo* biotinylated proteins were detected in this study.

When the ascorbate reduction step was omitted, GSNO-treated cells showed very low but detectable levels of biotinylation, similar to those observed in untreated or GSH-treated samples. In this assay, ascorbate is used selectively to reduce nitrosothiols in order that they can be labelled by biotin. However, this may also occur spontaneously during the biotinylation step, an effect observed in another study [26] which may explain the low levels of S-nitrosylation detected in the absence of ascorbate reduction.

Finally, the specificity of the assay for S-nitrosylation was verified by treating spermatozoa with oxidised GSH (GSSG) as it has been suggested that the biotin switch assay may also detect protein S-glutathionylation [41]. Only faint bands were detected with GSSG-dependent labelling (Figure 1B) similar to the ones observed in control samples. Taken together, these results confirm the specificity of the biotin switch method for detection of S-nitrosylated proteins in human spermatozoa.

The above experiments were carried out on spermatozoa exposed to non-capacitating conditions. We performed several experiments where the same procedures (detection and identification of S-nitrosylated proteins, localisation of S-nitrosylated proteins) were carried out on spermatozoa incubated under capacitating conditions but could not detect any consistent differences compared to spermatozoa incubated under non-capacitating conditions (data not shown). Thus, the results presented herein are from spermatozoa incubated under non-capacitating conditions.

3.2 Localisation S-Nitrosoproteins in Human Spermatozoa

Spermatozoa treated with GSNO or CSNO (≥25 μM) and labelled with MTSEA-Texas red showed bright fluorescence over the head, mid and principal piece of the tail but with the greatest intensities over the post-acosomal region of the sperm head (Figure 2 and 3). The pattern was similar in sperm treated with spermine NONOate (Figure 3) or with lower concentrations of GSNO or CSNO (data not shown) but staining in the post acrosomal region was less intense. By contrast, control slides (untreated or GSH-treated spermatozoa) showed faint fluorescence on the entire length of the sperm (head and tail; Figure 2 and 3). Fluorescence was reduced to control levels by the omission of ascorbate (data not shown)

confirming the specificity of this assay. In spermatozoa treated without MMTS to block thiol groups there was strong fluorescence throughout the entire cell (data not shown). To determine if the weak staining observed in control cells was due to endogenous S-nitrosylation we treated samples with the NOS-inhibitor, *N*(G)-nitro-L-arginine methyl ester hydrochloride (L-NAME) or with DTT. This failed to decrease the level of fluoresence suggesting that the fluorescence observed in controls was due to non-specific labelling (data not shown).

The general distribution of labelling is to be expected in view of the large number of S-nitrosylation targets identified (see Table 1; Supplementary Table S1). The more intense labelling produced by GSNO (Figure 2 and 3) and CSNO (data not shown) was probably because these agents can induce protein S-nitrosylation by transnitrosylation as well as by acting as NO donors. The former mechanism is likely to predominate since transnitrosylation reactions are faster than NO release [42]. By contrast, spermine NONOate acts as a pure NO donor and NO probably has to be oxidised to N_2O_3, a relatively slow reaction except at high NO concentrations [43] before it can react with thiols to produce S-nitrosothiols.

3.3 Proteomic Identification of S-Nitrosylated Protein from GSNO-Treated Human Spermatozoa

Following treatment of sperm by NO donors and the biotin switch assay, biotinylated proteins were affinity purified using streptavidin-agarose and proteins selectively eluted with DTT, which cleaved the disulphide bonds formed by biotin-HPDP. Proteins were then separated by SDS-PAGE, visualised by silver staining and the protein bands of interest excised from the gel, digested with trypsin, and analysed by MALDI-TOF mass spectrometry (Figure 4; Table 1; Supplementary Table S1).

In this study we have identified 240 proteins present in sperm from normozoospermic men which, in the presence of nitric oxide donors, undergo S-nitrosylation at cysteine residues. These proteins are listed in Table 1 and Supplementary Table S1. Comparison with the only publicly available human sperm proteome found only 30 (12%) in common [44]. An earlier study estimated that the human sperm proteome comprises at least 1760 proteins [45] indicating that the capacity of proteins to become S-nitrosylated upon exposure to NO is not ubiquitous; an essential characteristic of a modification if it is to have specific downstream effects. Analysis of both the cellular localisation and the cellular function of the identified proteins were performed. In both cases the largest group identified was that for which there was no information available in the Gene Ontology (GO) database; 23% and 22% of proteins, respectively. This is in keeping with the findings of the Johnson *et al.*, [45] and also with a recent microarray analysis of gene expression in the testis of a set of infertile men where, together “spermatogenesis” and “unknown” were the most significant GO categories enriched in germ cell genes [46].

The cellular distribution of the 240 identified proteins is shown in Figure 5A. Discounting those proteins with no associated gene ontology, the most commonly identified location was the cytoplasm (21%). This was somewhat surprising given that mature spermatozoa shed the majority of their cytoplasm in a residual body during spermiation. This most likely is a reflection of the morphological differences between sperm and somatic cells. Sperm are highly specialised cells which contain a number of unique, specialised organelles and it is likely that the cellular location of many proteins differs between sperm and other cell types. For example, in sperm many of the glycolytic enzymes are found in the flagellum compared to the cytoplasm in somatic cells [47]. It should also be noted that there is only a small amount of sperm-specific data in collections such as the Gene Ontology database. The next most populated compartments were cytoskeleton (13%), membrane (10%), mitochondria

(10%) and nucleus (8%). We saw an enrichment of membranous and nuclear proteins and a decrease in the proportion of mitochondrial proteins compared with the study of Martinez-Heredia *et al.* [44].

The functional analysis of the proteins identified is shown in Figure 5B. Metabolic proteins and protein associated with energy generation and cell movement were found to be abundant (22% when combined) suggesting a role for S-nitrosylation in sperm motility. Another abundant group of proteins were those involved in signal transduction (9%), fitting with a role for S-nitrosylation in the modulation of sperm function. Surprisingly, given that sperm are generally assumed to be transcriptionally inactive, 4% of the proteins that we identified as being S-nitrosylated were associated with transcription. The presence of proteins involved in transcription and protein synthesis has been previously observed in sperm proteomic studies [44, 48] and is further discussed in Lefièvre *et al.* [49]. We were interested to note that when we compared the human sperm S-nitrosoproteome with proteins identified during a proteomic analysis of sperm-egg interaction we found only 3 proteins in common suggesting that S-nitrosylation is not a regulatory mechanism employed during fertilisation [50].

We next interrogated the available literature to assess the proportion of the proteins that we identified which had previously been shown to be S-nitrosylated in other cells. Out of the 240 proteins we identified only 52 (22%) had been documented to undergo S-nitrosylation in other cell types. The remaining proteins have not yet been reported to be S-nitrosylated and as such represent a considerable addition to the number of identified target proteins modified by NO.

3.4 Biological significance

In this study we have demonstrated that a large number of human sperm proteins are potential targets for S-nitrosylation. Whilst it is important as a first step to determine the S-nitrosoproteome of a cell, three key questions must be addressed: 1) Are spermatozoa exposed to sufficient levels of NO *in vivo*? 2) How does S-nitrosylation affect bioactivity of sperm proteins? 3) What is the biological significance of sperm protein S-nitrosylation for fertilisation?

3.4.1 Are spermatozoa exposed to significant levels of NO *in vivo*?—It is known that sperm can synthesise NO, but the evidence that synthesis is sufficient to be physiologically significant is equivocal [6, 7]. Measured NO production in human sperm suspensions is decreased when in the presence of NOS inhibitors [11, 51] at doses that are reported to reduce sperm motility [8], serine-threonine and tyrosine phosphorylation [9, 52], acrosome reaction [9, 11] and sperm-oocyte fusion [53]. In contrast, it is well known that a number of cell types in the mammalian female reproductive tract, including oviductal cells, generate significant levels of NO [5] and our own studies using the fluorescent sensor DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) have shown that NO is present in human cumulus at far higher concentrations than in sperm suspensions (unpublished data). Immunolocalisation of eNOS and nNOS in cumulus cells suggest that the NO detected are produced by these cells and not from oocytes. Nitrosylation of sperm proteins *in vivo* is thus more likely to occur in response to NO generated by cells of the female tract than by autocrine effects of sperm NO generation. Recent studies that showed that one-by-one deletion of the three types of NOS (eNOS, nNOS, inducible, iNOS) in KO mice had no discernible effect on male or female fertility, save some positive effects of iNOS deletion [54], do not exclude an important role for NO in fertilisation because of possible compensation by other sources of NO and the high degree of redundancy in the regulatory cascades that control capacitation/fertilisation.

3.4.2 How does S-nitrosylation affect bioactivity of sperm proteins?—To understand fully the significance of our observations it will be necessary to investigate, for each S-nitrosylated protein, whether S-nitrosylation alters protein bioactivity, to what degree, its reversibility and which cysteine (s) are involved. For spermatozoa, this is likely to involve *in vitro* transfection of somatic cells with specific proteins e.g. AKAPs in order to obtain a detailed understanding. However, a number of proteins merit discussion because of their known significance in sperm biology.

A-Kinase anchoring proteins (AKAPs): AKAPs have never before been shown to be S-nitrosylated. AKAP3 (O75969, Table 1) and AKAP4 (Q5JQC9, Table 1) are believed to localise signalling complexes in the spermatozoa but, compared to their functional significance in other cell types relatively little is know [55, 56]. AKAP4 is a major protein present in the fibrous sheath of the sperm tail which binds both PRKAR1 (R1α) and PRKAR2A (R11α) regulatory subunit of PKA and is one of the key proteins phosphorylated during the process of capacitation [2, 57]. AKAP3 is also present in the fibrous sheath. In bovine sperm AKAP3 binds to PDE4A isoforms, potentially controlling PKA activity [58], and is tyrosine phosphorylated during capacitation in humans. AKAP complexes in the sperm tail modulate the motility of the cells. For example, specific inhibitors of PDE's (RS23544 –PDE4A5 inhibitor; Fisch *et al.* [59] noticeably increase the motility of the cell and protein kinase A-anchoring inhibitor peptides arrest sperm motility [60].

Our identification of three sperm AKAP proteins as being capable of undergoing S-nitrosylation is further supported by the presence of a consensus S-nitrosylation motif in their primary protein sequences. The acid-Cys-base motif was reported by Stamler *et al.* as being present, initially in haemoglobin [61], and subsequently in a number of other proteins known to undergo S-nitrosylation *in vivo* [62]. The redox environment need not necessarily to be created by acidic and basic residues adjacent to the target cysteine residue in the primary sequence and may instead be placed in close proximity in the tertiary structures [63]. In a number of proteins the presence of a Cys-(Asp,Glu) motif appears to be sufficient [62]. A role for further polar amino acid residues in the vicinity of the modified cysteine has also been proposed [61]. In the context of the well documented effects of NO on sperm motility, S-nitrosylation of AKAP's presents a novel form of modulation which should be the focus of further studies.

Heat shock proteins (HSPs): In this study we showed that a number of heat shock proteins, belonging to the major 90kDa, 70kDa and 60kDa families, were S-nitrosylated. A number of these have recently been reported to play a very significant role in sperm capacitation and determination of fertilising ability. For example; heat shock protein 1 (chaperonin; heat shock protein 60kDa family) (homologue of human 60 kDa heat shock protein; P10809, Table 1) and endoplasmin (ERP99; heat shock protein 90kDa family) (homologue of human endoplasmin; P10809, Table 1) are tyrosine phosphorylated during capacitation in mouse sperm [64] where it is suggested that these proteins form part of a zona receptor complex during capacitation allowing the successful binding of the sperm to the egg [65]. Heat shock 70 kDa protein 8 (P11142, Table 1) and heat shock protein 90α (P7900, Table 1) are tyrosine phosphorylated during capacitation in human spermatozoa [2] but whether they function in a zona receptor complex is as yet unknown. The bovine homologue of heat shock protein 1 (HSP60) was found to become associated with the sperm surface during transit through the oviduct and is suggested to have a role in sperm capacitation [66]. HspA2 (also known as heat shock-related 70 kDa protein 2) (P54652, Table 1) has been shown to be a marker of sperm maturity [67] with lower levels of HspA2 gene expression in oligoteratoasthenozoospermic men [68]. We have found S-nitrosylation to be a common modification of heat shock proteins in human spermatozoa. This has also been showed in

other cell types (see Table 1) suggesting that S-nitrosylation may be a universal mechanism for the modification of heat shock protein function.

Ryanodine receptors (RyRs): RyRs are intracellular Ca^{2+} channels that play a central role in $[Ca^{2+}]_i$ signalling, particularly signal amplification by Ca^{2+}-induced Ca^{2+} release. These proteins contain a large number of sulfhydryls and are thus subject to S-nitrosylation [69, 70]. NO or NO-related proteins modulate activity of RyR1 and RyR2 [71-74], critical SH residues in RyR1 having been identified [73, 75] and NO donors rapidly mobilise Ca^{2+} from skeletal muscle and cardiac microsomes, an effect that can be reversed by thiol reducing agents [76]. In the present study we have identified RyR2 by MS/MS as being S-nitrosylated by NO treatment of human spermatozoa (Q92736, Table 1). The literature on expression of RyRs in the male gamete is inconsistent. Both the presence and (when detected) type of RyRs in mouse germ cells is controversial [77-79]. No RyR expression was detected with antibodies or with a fluorescent analogue of ryanodine (BODIPY-FL-X-ryanodine) in bull sperm [80]. However, BODIPY-FL-X-ryanodine stains live human sperm, primarily in the area of the sperm neck and mid-piece section of the flagellum [81]. Immunolocalisation using polyclonal antibodies specific for RyR1 and RyR2 confirm the presence of both receptors in the neck and mid-piece region of the human spermatozoa (unpublished data). Cyclic mobilisation of stored Ca^{2+} in this region, which regulates flagellar activity, is sensitive to caffeine and ryanodine [81]. Our findings confirm the presence of RyRs in human sperm but the low incidence of detection (Table 1, Supplementary Table S1) suggests that they may be present in low abundance (which may partly explain the difficulty in their detection and characterisation). This is consistent with their restricted cellular localisation [81] and might be expected for expression of a high conductance ion channel in a cell with a very small cytoplasmic volume. Significantly, the ryanodine-sensitive Ca^{2+}-store in human sperm is mobilised upon exposure to NO [82] (Machado-Oliveira and Publicover, personal communication).

Glycolytic enzymes: It is interesting that a number of glycolytic enzymes are targets for S-nitrosylation (see Table 1). Human sperm require glucose (or some other glycolysable sugar) for optimum motility although unlike mouse sperm they do not require it for capacitation [83]. The sperm-specific isoform of glyceraldehyde 3-phosphate dehydrogenase (GAPDHs; O14556, Table 1) is of particular interest. This enzyme is bound to the fibrous sheath of the flagellum [84] and is required for sperm motility in the mouse [85]. GAPDHs has a very similar primary sequence to the somatic isoenzymes with the addition of a proline rich N-terminal extension [86]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; P04406, Table 1) is rich in sulphydryl groups and is a known target for S-nitrosylation. S nitrosylated GAPDH has decreased enzymatic activity and as part of the cellular response to NO stress, it associates with Siah (an E3 ubiquitin lyase) and the complex is transported to the nucleus where it promotes apoptosis [87]. Since GAPDHs is anchored to the fibrous sheath it will be unable to act in this way but nitrosylation could affect its interaction with other fibrous sheath proteins. Another interesting enzyme is sperm specific lactate dehydrogenase (LDH-C4; P07864, Table 1). Alone among lactate dehydrogenase isoforms it is expressed in the mitochondrion as well as the cytoplasm offering the sperm a unique lactate-pyruvate redox shuttle [88].

3.4.3 What is the biological significance of sperm protein S-nitrosylation for fertilisation?—

We need to understand the physiological importance of S-nitrosylation modification in regulating the competence of sperm to fertilise. Although poorly understood, the movement of sperm through the female reproductive tract is known to be a finely controlled, complex and dynamic process [1, 89]. During most of this journey, the female reproductive tract ‘holds back’ the sperm from being ready to fertilise, yet, as the sperm

approach the egg they have to become fully competent to fertilise, which includes increasing the 'power' of the cell so that it can penetrate the cumulus and the zona pellucida [90]. The cumulus is a logical source of external signals to activate sperm prior to fertilisation *in vivo* and is a likely source of NO which would have a maximal effect on the sperm. It is our hypothesis that sperm will experience a modest tonic NO stimulus from endogenous generation (and possible sources in the female tract) but encounter a steeply increasing concentration of NO on entering the cumulus [17, 82]. Further experiments are warranted to test this concept.

4. Concluding Remarks

Despite the well known effects of NO on human spermatozoa, the identification of S-nitrosylated proteins until now remained unexplored. In this study, using a series of NO donors combined with the biotin switch assay we report the identification of S-nitrosylated proteins in human spermatozoa. This global assessment allows critical insight into the role of NO in sperm physiology identifies potential novel mechanisms of action of S-nitrosylation and opens up a new signalling regimen in the spermatozoon which will act as a platform for further detailed studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge the donors who took part in our research and the staff in the Assisted Conception Unit for assistance in collecting semen samples. We would also like to thank Prof F.A. Lai (Cardiff University) for the polyclonal anti-RyR1 (2142) and anti-RyR2 (1093) antibodies. This study was supported Wellcome Trust Grant 078905 [L.L, C.L.R.B.], Fonds de la recherche en santé du Québec [L.L.] and the Functional Genomics Laboratory.

Abbreviations

AKAP	A-kinase anchor proteins
Biotin-HPDP	N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide
CSNO	L-nitroso-cysteine
DAF-FM diacetate	4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate
ERKs	Protein extracellular signal regulated kinases
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAPDHs	sperm isoform specific glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione
GSNO	S-nitroso-glutathione
GSSG	oxidised glutathione
HSPs	Heat shock proteins
iNOS	inducible nitric oxide synthase
LDH-C4	sperm specific lactate dehydrogenase

L-NAME	N(G)-Nitro-L-arginine methyl ester
MMTS	Methanethiosulfonate
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
NOS	nitric oxide synthase
TEXAS RED®-MTSEA	Texas Red®-2-sulfonamidoethyl methanethiosulfonate
TTBS	Tris-buffered saline supplemented with 0.1% Tween-20
PKA	protein kinase A
PDE	phosphodiesterase
RyR	ryanodine receptor
Spermine NONOate	N-(2-aminoethyl)-N-(2-hydroxy-nitrosohydrazino)-1,2-ethylenediamine

5. References

1. Suarez SS, Pacey AA. Hum. Reprod. Update. 2006; 12:23–37. [PubMed: 16272225]
2. Ficarro S, Chertihin O, Westbrook VA, White F, et al. J. Biol. Chem. 2003; 278:11579–11589. [PubMed: 12509440]
3. Ford WC. Hum. Reprod. Update. 2004; 10:387–399. [PubMed: 15218008]
4. Baker MA, Aitken RJ. Mol. Cell. Endocrinol. 2004; 216:47–54. [PubMed: 15109744]
5. Rosselli M, Keller PJ, Dubey RK. Hum. Reprod. Update. 1998; 4:3–24. [PubMed: 9622410]
6. Herrero MB, Gagnon C. J. Androl. 2001; 22:349–356. [PubMed: 11330633]
7. Herrero MB, de Lamirande E, Gagnon C. Curr. Pharm. Des. 2003; 9:419–425. [PubMed: 12570819]
8. Lewis SEM, Donnelly ET, Sterling ESL, Kennedy MS, et al. Mol. Hum. Reprod. 1996; 2:873–878. [PubMed: 9237228]
9. Herrero MB, de Lamirande E, Gagnon C. Biol. Reprod. 1999; 61:575–581. [PubMed: 10456831]
10. Sengoku K, Tamate K, Yoshida T, Takaoka Y, et al. Fértil. Steril. 1998; 69:522–527.
11. Revelli A, Soldati G, Costamagna C, Pellerey O, et al. J. Cell. Physiol. 1999; 178:85–92. [PubMed: 9886494]
12. Willipinski-Stapelfeldt B, Lubberstedt J, Stelter S, Vogt K, et al. Mol. Hum. Reprod. 2004; 10:543–552. [PubMed: 15121877]
13. Zhang H, Zhou QM, Li XD, Xie Y, et al. Arch. Pharm. Res. 2006; 29:145–151. [PubMed: 16526279]
14. Herrero MB, Perez Martinez S, Viggiano JM, Polar JM, de Gimeno MF. Reprod. Fertil. Dev. 1996; 8:931–934. [PubMed: 8876053]
15. O'Bryan MK, Zini A, Cheng CY, Schlegel PN. Fertil. Steril. 1998; 70:1143–1147. [PubMed: 9848308]
16. Marcondes S, Cardoso MH, Morganti RP, Thomazzi SM, et al. Proc. Natl. Acad. Sci. USA. 2006; 103:3434–3439. [PubMed: 16492779]
17. Publicover SJ, Harper CV, Barratt CLR. Nat. Cell. Biol. 2007; 9:235–242. [PubMed: 17330112]
18. Revelli A, Ghigo D, Moffa F, Massobrio M, Tur-Kaspa I. Endocr. Rev. 2002; 23:484–494. [PubMed: 12202462]
19. Lefièvre L, De Lamirande E, Gagnon C. J. Androl. 2000; 21:929–937. [PubMed: 11105920]
20. Bielfeld P, Faridi A, Zaneveld LJ, De Jonge CJ. Fertil. Steril. 1994; 61:536–541. [PubMed: 8137980]

21. Herrero MB, Chatterjee S, Lefièvre L, de Lamirande E, Gagnon C. Free Radic. Biol. Med. 2000; 29:522–536. [PubMed: 11025196]
22. Thundathil J, de Lamirande E, Gagnon C. Biol. Reprod. 2003; 68:1291–1298. [PubMed: 12606410]
23. O'Flaherty C, de Lamirande E, Gagnon C. Free Radic. Biol. Med. 2006; 40:1045–1055. [PubMed: 16540400]
24. Stamler JS, Simon DI, Osborne JA, Mullins ME, et al. Proc. Natl. Acad. Sci. USA. 1992; 89:444–448. [PubMed: 1346070]
25. Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Nat. Rev. Mol. Cell Biol. 2005; 6:150–166. [PubMed: 15688001]
26. Foster MW, Stamler JS. J. Biol. Chem. 2004; 279:25891–25897. [PubMed: 15069080]
27. Greco TM, Hodara R, Parastatidis I, Heijnen HF, et al. Proc. Natl. Acad. Sci. USA. 2006; 103:7420–7425. [PubMed: 16648260]
28. Hao G, Derakhshan B, Shi L, Campagne F, Gross SS. Proc. Natl. Acad. Sci. USA. 2006; 103:1012–1017. [PubMed: 16418269]
29. Wang G, Moniri NH, Ozawa K, Stamler JS, Daaka Y. Proc. Natl. Acad. Sci. USA. 2006; 103:1295–1300. [PubMed: 16432212]
30. Rhee KY, Erdjument-Bromage H, Tempst P, Nathan CF. Proc. Natl. Acad. Sci. USA. 2005; 102:467–472. [PubMed: 15626759]
31. Garban HJ, Marquez-Garban DC, Piedras RJ, Ignarro LJ. Proc. Natl. Acad. Sci. USA. 2005; 102:2632–2636. [PubMed: 15699347]
32. Retamal MA, Cortes CJ, Reuss L, Bennett MV, Saez JC. Proc. Natl. Acad. Sci. USA. 2006; 103:4475–4480. [PubMed: 16537412]
33. World Health Organization Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction. 4th edn. Cambridge University Press; Cambridge: 1999.
34. Kvist, U.; Björndahl, L. Manual on Basic Semen Analysis. ESHRE Monographs #2. Oxford University Press; Oxford: 2002.
35. Jacobson G, Karsnas P. Electrophoresis. 1990; 11:46–52. [PubMed: 1690643]
36. Yang Y, Loscalzo J. Proc. Natl. Acad. Sci. USA. 2005; 102:117–122. [PubMed: 15618409]
37. Pixton KL, Deeks ED, Flesch FM, Moseley FL, et al. Hum. Reprod. 2004; 19:1438–1447. [PubMed: 15105389]
38. Lefièvre L, Conner SJ, Salpekar A, Olufowobi O, et al. Hum. Reprod. 2004; 19:1580–1586. [PubMed: 15142998]
39. Kinter, M.; Sherman, NE. Protein Sequencing and Identification Using Tandem Mass Spectrometry. John Wiley; New York: 2000.
40. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Nat. Cell Biol. 2001; 3:193–197. [PubMed: 11175752]
41. Kuncewicz T, Sheta EA, Goldknopf IL, Kone BC. Mol. Cell. Proteomics. 2003; 2:156–163. [PubMed: 12671063]
42. Arnelle DR, Stamler JS. Arch. Biochem. Biophys. 1995; 318:279–285. [PubMed: 7733655]
43. Gow AJ, Ischiropoulos H. J. Cell. Physiol. 2001; 187:277–282. [PubMed: 11319751]
44. Martinez-Heredia J, Estanyol JM, Ballesca JL, Oliva R. Proteomics. 2006; 6:4356–4369. [PubMed: 16819732]
45. Johnston DS, Wooters J, Kopf GS, Qiu Y, Roberts KP. Ann. N. Y. Acad. Sci. 2005; 1061:190–202. [PubMed: 16467268]
46. Ellis PJI, Furlong RA, Conner SJ, Kirkman-Brown JC, Afnan M, Barratt CLR, Griffin DK, Affara NA. Hum. Mol. Genet. Submitted.
47. Eddy EM, Toshimori K, O'Brien DA. Microsc. Res. Tech. 2003; 61:103–115. [PubMed: 12672126]
48. Lalancette C, Faure RL, Leclerc P. Proteomics. 2006; 6:4523–4540. [PubMed: 16847872]
49. Lefièvre L, Bedu-Addo K, Conner SJ, Machado-Oliveira GSM, et al. Reproduction. In Press.

50. Stein KK, Go JC, Lane WS, Primakoff P, Myles DG. Proteomics. 2006; 6:3533–3543. [PubMed: 16758446]
51. Donnelly ET, Lewis SEM, Thompson W, Chaakravarthy U. Mol. Hum. Reprod. 1997; 3:755–762. [PubMed: 9358000]
52. O'Flaherty C, de Lamirande E, Gagnon C. Mol. Hum. Reprod. 2004; 10:355–363. [PubMed: 14997001]
53. Francavilla F, Santucci R, Macerola B, Ruvolo G, Romano R. Biol. Reprod. 2000; 63:425–429. [PubMed: 10906046]
54. Yang JZ, Ajonuma LC, Rowlands DK, Tsang LL, Ho LS, et al. Cell Biol. Int. 2005; 29:785–791. [PubMed: 16087361]
55. Smith FD, Langeberg LK, Scott JD. Trends Biochem. Sci. 2006; 31:316–323. [PubMed: 16690317]
56. McConnachie G, Langeberg LK, Scott JD. Trends Mol. Med. 2006; 12:317–323. [PubMed: 16809066]
57. Visconti PE, Johnson LR, Oyaski M, Fornes M, et al. Dev. Biol. 1997; 192:351–363. [PubMed: 9441673]
58. Bajpai M, Fiedler SE, Huang Z, Vijayaraghavan S, et al. Biol. Reprod. 2006; 74:109–118. [PubMed: 16177223]
59. Fisch JD, Behr B, Conti M. Hum. Reprod. 1998; 13:1248–1254. [PubMed: 9647555]
60. Vijayaraghavan S, Goueli SA, Davey MP, Carr DW. J. Biol. Chem. 1997; 272:4747–4752. [PubMed: 9030527]
61. Stamler JS, Toone EJ, Lipton SA, Sucher NJ. Neuron. 1997; 18:691–696. [PubMed: 9182795]
62. Stamler JS. Cell. 1994; 78:931–936. [PubMed: 7923362]
63. Perez-Mato I, Castro C, Ruiz FA, Corrales FJ, Mato JM. J. Biol. Chem. 1999; 274:17075–17079. [PubMed: 10358060]
64. Asquith KL, Baleato RM, McLaughlin EA, Nixon B, Aitken RJ. J. Cell Sci. 2004; 117:3645–3657. [PubMed: 15252132]
65. Nixon B, Asquith KL, Aitken RJ. Mol. Cell. Endocrinol. 2005; 240:1–10. [PubMed: 16043280]
66. Boilard M, Reyes-Moreno C, Lachance C, Massicotte L, et al. Biol. Reprod. 2004; 71:1879–1889. [PubMed: 15286042]
67. Ergur AR, Dokras A, Giraldo JL, Habana A, et al. Fertil. Steril. 2002; 77:910–918. [PubMed: 12009343]
68. Cedenho AP, Lima SB, Cenedeze MA, Spaine DM, et al. Hum. Reprod. 2006; 21:1791–1794. [PubMed: 16517558]
69. Takeshima H, Nishimura S, Matsumoto T, Ishida H, et al. Nature. 1989; 339:439–445. [PubMed: 2725677]
70. Otsu K, Willard HF, Khanna VK, Zorzato F, et al. J. Biol. Chem. 1990; 265:13472–13483. [PubMed: 2380170]
71. Xu L, Eu JP, Meissner G, Stamler JS. Science. 1998; 279:234–237. [PubMed: 9422697]
72. Zahradnikova A, Minarovic I, Venema RC, Meszaros LG. Cell Calcium. 1997; 22:447–454. [PubMed: 9502194]
73. Sun J, Xin C, Eu JP, Stamler JS, Meissner G. Proc. Natl. Acad. Sci. USA. 2001; 98:11158–11162. [PubMed: 11562475]
74. Sun J, Xu L, Eu JP, Stamler JS, Meissner G. J. Biol. Chem. 2003; 278:8184–8189. [PubMed: 12509428]
75. Aracena-Parks P, Goonasekera SA, Gilman C, Dirksen RT, et al. J. Biol. Chem. 2006; 281:40354–40368. [PubMed: 17071618]
76. Stoyanovsky D, Murphy T, Anno PR, Kim YM, Salama G. Cell Calcium. 1997; 21:19–29. [PubMed: 9056074]
77. Giannini G, Conti A, Mammarella S, Scrobogna M, Sorrentino V. J. Cell Biol. 1995; 128:893–904. [PubMed: 7876312]

78. Trevino CL, Santi CM, Beltran C, Hernandez-Cruz A, et al. Zygote. 1998; 6:159–172. [PubMed: 9770782]
79. Chiarella P, Puglisi R, Sorrentino V, Boitani C, Stefanini M. J. Cell Sci. 2004; 117:4127–4134. [PubMed: 15280431]
80. Ho HC, Suarez SS. Biol. Reprod. 2001; 65:1606–1615. [PubMed: 11673282]
81. Harper CV, Barratt CL, Publicover SJ. J. Biol. Chem. 2004; 279:46315–46325. [PubMed: 15322137]
82. Conner SJ, Lefièvre L, Kirkman-Brown JC, Michelangeli F, et al. Soc. Reprod. Suppl. 2007; 63:237–256.
83. Williams AC, Ford WC. J. Androl. 2001; 22:680–695. [PubMed: 11451366]
84. Eddy EM, Toshimori K, O'Brien DA. J. Androl. 2003; 22:680–695.
85. Miki K, Qu W, Goulding EH, Willis WD, et al. Proc. Natl. Acad. Sci. USA. 2004; 101:16501–16506. [PubMed: 15546993]
86. Welch JE, Schatte EC, O'Brien DA, Eddy EM. Biol. Reprod. 1992; 46:869–878. [PubMed: 1375514]
87. Hara MR, Cascio MB, Sawa A. Biochim. Biophys. Acta. 2006; 1762:502–509. [PubMed: 16574384]
88. Ford, WCL.; Rees, JM. Controls of sperm motility: biological and clinical aspects. Gagnon, C., editor. CRC Press; Boca Raton: 1990. p. 175-202.
89. De Jonge C. Hum. Reprod. Update. 2005; 11:205–214. [PubMed: 15817522]
90. Quill TA, Sugden SA, Rossi KL, Doolittle LK, et al. Proc. Natl Acad Sci U S A. 2003; 100:14869–14874. [PubMed: 14657366]
91. Martinez-Ruiz A, Lamas S. Arch. Biochem. Biophys. 2004; 423:192–199. [PubMed: 14871481]
92. Stamler JS, Lamas S, Fang FC. Cell. 2001; 106:675–683. [PubMed: 11572774]
93. Lee M, Arosio P, Cozzi A, Chasteen ND. Biochemistry. 1994; 33:3679–3687. [PubMed: 8142366]
94. Nedospasov A, Rafikov R, Beda N, Nudler E. Proc. Natl. Acad. Sci. USA. 2000; 97:13543–13548. [PubMed: 11095728]
95. Stamler JS, Jaraki O, Osborne J, Simon D, et al. Proc. Natl. Acad. Sci. USA. 1992a; 89:7674–7677. [PubMed: 1502182]
96. Stamler JS, Simon DI, Osborne JA, Mullins ME, et al. Proc. Natl. Acad. Sci. USA. 1992c; 89:444–448. [PubMed: 1346070]
97. Belluoccio D, Wilson R, Thornton DJ, Wallis TP, et al. Proteomics. 2006; 6:6549–6553. [PubMed: 17163436]
98. Gao C, Guo H, Wei J, Mi Z, et al. Nitric Oxide. 2005; 12:121–126. [PubMed: 15740986]
99. Lind C, Gerdes R, Hamnell Y, Schuppe-Koistinen I, et al. Arch. Biochem. Biophys. 2002; 406:229–240. [PubMed: 12361711]
100. Lindermayr C, Saalbach G, Durner J. Plant Physiol. 2005; 137:921–930. [PubMed: 15734904]
101. Dall'Agnol M, Bernstein C, Bernstein H, Garewal H, Payne CM. Proteomics. 2006; 6:1654–1662. [PubMed: 16404723]
102. Moon KH, Hood BL, Kim BJ, Hardwick JP, et al. Hepatology. 2006; 44:1218–1230. [PubMed: 17058263]
103. Fratelli M, Demol H, Puype M, Casagrande S, et al. Proc. Natl. Acad. Sci. USA. 2002; 99:3505–3510. [PubMed: 11904414]
104. Fratelli M, Demol H, Puype M, Casagrande S, et al. Proteomics. 2003; 3:1154–1161. [PubMed: 12872216]
105. Shenton D, Grant CM. Biochem. J. 2003; 374:513–519. [PubMed: 12755685]
106. Ji Y, Akerboom TP, Sies H, Thomas JA. Arch. Biochem. Biophys. 1999; 362:67–78. [PubMed: 9917330]
107. Klatt P, Pineda Molina E, Perez-Sala D, Lamas S. Biochem. J. 2000; 349:567–578. [PubMed: 10880356]
108. Ito H, Iwabuchi M, Ogawa K. Plant Cell Physiol. 2003; 44:655–660. [PubMed: 12881492]

109. Molina y Vedia L, McDonald B, Reep B, Brune B, et al. J. Biol. Chem. 1992; 267:24929–24932. [PubMed: 1281150]
110. Padgett CM, Whorton AR. Am. J. Physiol. 1995; 269:C739–749. [PubMed: 7573405]
111. Mohr S, Stamler JS, Brune B. J. Biol. Chem. 1996; 271:4209–4214. [PubMed: 8626764]
112. Mohr S, Hallak H, de Boitte A, Lapetina EG, Brune B. J. Biol. Chem. 1999; 274:9427–9430. [PubMed: 10092623]
113. Motohashi K, Kondoh A, Stumpp MT, Hisabori T. Proc. Natl. Acad. Sci. USA. 2001; 98:11224–11229. [PubMed: 11553771]
114. Hara MR, Agrawal N, Kim SF, Cascio MB, et al. Nat. Cell Biol. 2005; 7:665–674. [PubMed: 15951807]
115. Yamazaki D, Motohashi K, Kasama T, Hara Y, Hisabori T. Plant Cell Physiol. 2004; 45:18–27. [PubMed: 14749482]
116. Stamler JS, Simon DI, Jaraki O, Osborne JA, et al. Proc. Natl. Acad. Sci. USA. 1992b; 89:8087–8091. [PubMed: 1325644]
117. Eaton P, Jones ME, McGregor E, Dunn MJ, et al. J. Am. Soc. Nephrol. 2003; 14:S290–296. [PubMed: 12874448]
118. DeMaster EG, Redfern B, Quast BJ, Dahlseid T, Nagasawa HT. Alcohol. 1997; 14:181–189. [PubMed: 9085720]
119. Hmadcha A, Bedoya FJ, Sobrino F, Pintado E. J. Exp. Med. 1999; 190:1595–1604. [PubMed: 10587350]
120. Laval F, Wink DA. Carcinogenesis. 1994; 15:443–447. [PubMed: 8118926]

Europe PMC Funders Author Manuscripts

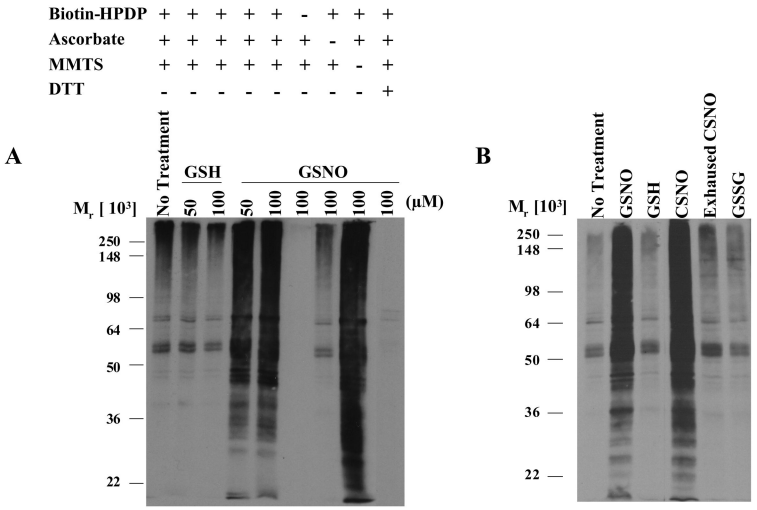

Figure 1. Detection of S-nitrosylated proteins in human spermatozoa with biotin switch assay

Intact human sperm were treated with GSNO, GSH, CSNO, exhausted CSNO or oxidised GSH (GSSG) or incubated with no treatment (no treatment) for 1hr at 37°C prior biotin switch assay as described in Materials and Methods. A series of controls were performed to assess the specificity of the biotin switch assay. A) Firstly where indicated, the assay was tested in the absence of biotin-HPDP to show if any endogenously biotinylated proteins could be detected. Secondly where indicated, labelling with biotin-HPDP was performed with or without ascorbate, MMTS or in presence of DTT. B) GSSG was used to assess that the assay was specific for S-nitrosylation and not S-glutathionylation. Proteins were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membrane. Detection of biotinylated proteins was achieved using anti-biotin antibody. The relative masses of protein standards are shown on the left. Results of 1 experiment presented are representative of 6 others performed with different sperm donors.

Europe PMC Funders Author Manuscripts

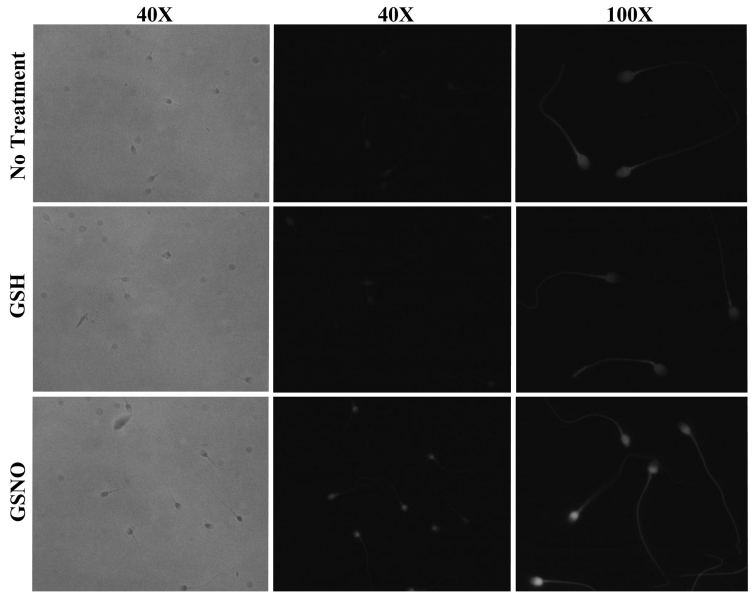

Figure 2. ***In situ*** **detection of S-nitrosylated targets in human spermatozoa**

Spermatozoa were incubated without (no treatment; top) or with GSH (middle) or GSNO (bottom) for 1 hr prior S-nitrosylated proteins labelling with Texas-red fluorescence-MTSEA as described in Materials and Methods. Left and middle columns represent phase and fluorescence images, respectively, using the 40X objective. On the right column, a more detailed localisation of sperm S-nitrosylation labelling is shown using 100X objective. Results of 1 experiment presented are representative of 6 others performed with different sperm donors.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

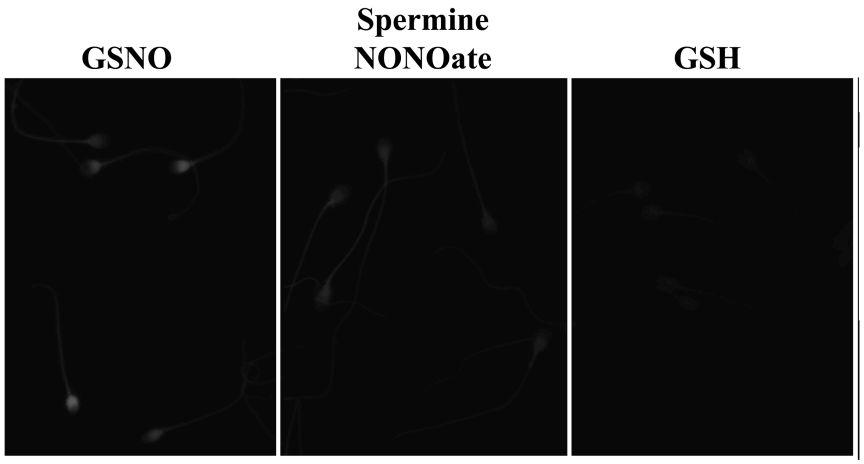

Figure 3. ***In situ*** **detection of S-nitrosylated targets using different nitric oxide donors**

Spermatozoa were incubated with GSH (top), spermine NONOate (middle) or GSNO (bottom) for 1 hr prior S-nitrosylated proteins labelling with Texas-red fluorescence-MTSEA as described in Materials and Methods. Fluorescence images were obtained using using 100X objective. Results of 1 experiment presented are representative of 3 others performed with different sperm donors.

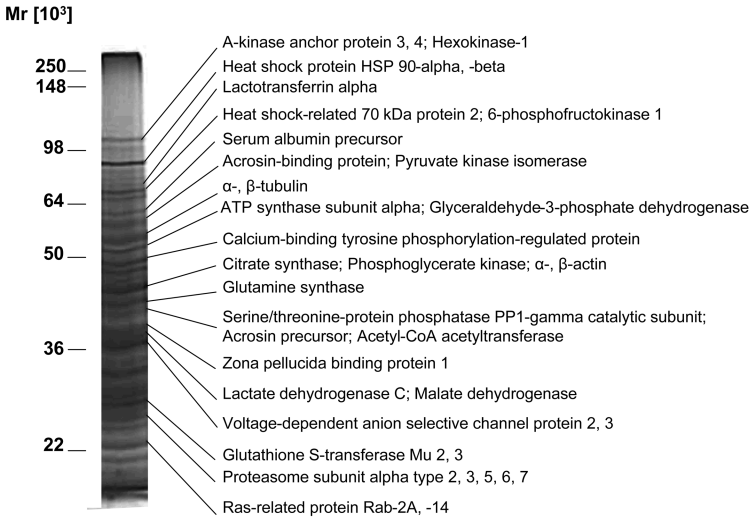

Figure 4. Isolation and identification of S-nitrosoproteins in human spermatozoa

Biotinylated sperm proteins obtained from the biotin switch assay were purified by affinity chromatography using streptavidin-agarose and eluted with sample buffer containing DTT as described in Materials and Methods. Proteins were separated by SDS-PAGE (10%) and visualized by silver staining. The relative masses of protein standards are shown on the left. Individual bands were trypsinised and submitted to MS/MS for protein identification. More than 1 protein was found for each bands (see Table 1). Results of 1 experiment are representative of 5 others performed with different pools of sperm donors. Proteins shown on the right side of the gel are a sample of known sperm proteins that are shown in Table 1.

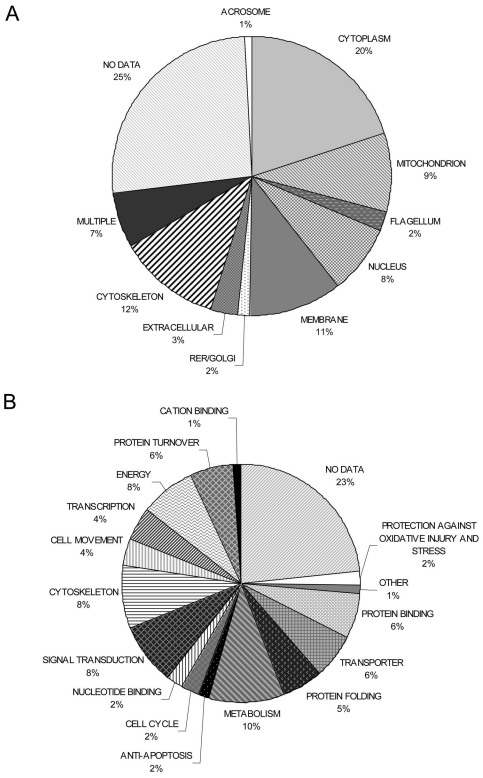

Figure 5. Ontology of the proteins identified as being S-nitrosylated in human spermatozoa
A) Distribution of the proteins in the different compartments of the spermatozoon. B) Biological function of the proteins.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Table 1

Human Sperm S-Nitrosoproteome[a,b]

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Signalling/Regulating Proteins							
Leucine-rich repeat protein SHOC-2	SHOC2_HUMAN	Q9UQ13	65.3	3	5.0	4	
Phosphoinositide 3-kinase regulatory subunit 5	PI3R5_HUMAN	Q8WYR1	98.6	2	1.1	1	
Protein kinase A-anchoring protein 3	AKAP3_HUMAN	O75969	95.4	16	25.8	3	
Protein kinase A-anchoring protein 4	AKAP4_HUMAN	Q5JQC9	95.8	28	39.9	3	
Protein kinase A-anchoring protein 9	AKAP9_HUMAN	Q99996	455.7	2	0.5	2	
14-3-3 protein zeta/delta	1433Z_HUMAN	P63104	27.9	3	17.1	1	[27, 28, 41, 91]
FKBP12-rapamycin complex-associated protein	FRAP_HUMAN	P42345	290.8	2	0.7	1	
Ras GTPase-activating-like protein IQGAP2	IQGA2_HUMAN	Q13576	180.9	3	1.8	1	
Ras GTPase-activating protein 4	RASL2_HUMAN	O43374	91.4	3	3.9	2	
Ras-related protein Rab-2A	RAB2A_HUMAN	P61019	23.7	3	22.2	1	
Ras-related protein Rab-14	RAB14_HUMAN	P61106	23.7	2	9.3	1	
Ribosome-binding protein 1	RRBP1_HUMAN	Q9P2E9	152.8	2	1.7	1	
Signal-regulatory protein gamma precursor	SIRPG_HUMAN	Q9P1W8	42.9	3	8.8	1	
Protein Kinases/Phosphatases							
cAMP-dependent protein kinase, alpha-catalytic subunit	KAPCA_HUMAN	P17612	40.5	2	6.3	1	
cAMP-dependent protein kinase type II-alpha regulatory subunit	KAP2_HUMAN	P13861	45.7	3	8.4	1	
Obscurin-myosin light chain kinase	OBSCN_HUMAN	Q5VST9	879.6	2	0.2	1	
Protein phosphatase 1 catalytic subunit gamma isoform		AAX29836.1	37.7	1	3.7	1	
Proto-oncogene tyrosine-protein kinase Yes	YES_HUMAN	P07947	61.1	2	3.3	1	
Serine/threonine-protein kinase ATR	ATR_HUMAN	Q13535	304.8	3	1.5	1	

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Serine/threonine-protein kinase 35	STK35_HUMAN	Q8TDR2	44.9	3	7.5	1	
Serine/threonine-protein kinase SMG1	SMG1_HUMAN	Q96Q15	414.1	2	0.6	1	
Tyrosine-protein kinase JAK1	JAK1_HUMAN	P23458	133.6	2	1.8	1	
Receptors							
Roundabout homolog 4 precursor	ROBO4_HUMAN	Q8WZ75	108.4	2	2.5	1	
Ryanodine receptor 2	RYR2_HUMAN	Q92736	569.3	3	0.7	1	[71, 76, 92]
Transport							
Alpha-centractin	ACTZ_HUMAN	P61163	42.7	3	9.8	1	
Choline transporter-like protein 1	CTL1_HUMAN	Q8WWI5	74.8	2	4.6	1	
Ferritin, mitochondrial precursor	FTMT_HUMAN	Q8N4E7	27.8	2	10.3	1	[92, 93]
Nucleoprotein TPR	TPR_HUMAN	P12270	265.8	3	1.3	1	
Probable phospholipid-transporting ATPase IA	AT8A1_HUMAN	Q9Y2Q0	132.6	2	1.7	1	
Serum albumin precursor	ALBU_HUMAN	P02768	71.3	10	18.6	2	[92, 94-97]
Sodium channel protein type 2 subunit alpha	SCN2A_HUMAN	Q99250	230.0	2	0.9	1	
Sodium channel protein type 4 subunit alpha	SCN4A_HUMAN	P35499	210.1	1	0.6	3	
Sodium channel protein type 5 subunit alpha	SCN5A_HUMAN	Q14524	229.4	1	0.6	3	
Solute carrier family 2, facilitated glucose transporter member 3	GTR3_HUMAN	P11169	54.3	2	1.8	2	
Solute carrier organic anion transporter family member 1B1	SO1B1_HUMAN	Q9Y6L6	77.5	2	3.2	2	
Voltage-dependent anion-selective channel protein 2	VDAC2_HUMAN	P45880	38.6	1	4.3	2	
Voltage-dependent anion-selective channel protein 3	VDAC3_HUMAN	Q9Y277	31.0	1	3.5	1	
Structural Proteins							
Actin, aortic smooth muscle	ACTA_HUMAN	P62736	42.4	6	20.7	2	
Actin, cytoplasmic 1 (Beta-actin)	ACTB_HUMAN	P60709	42.1	4	12.5	1	[40, 91, 97, 98]
Actin-related protein T2	ACTT2_HUMAN	Q8TDY3	42.1	3	5.8	1	

Protein Name	Swiss-Prot Entry Name	Swiss-Prot Accession No.	Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
Axonemal dynein light intermediate polypeptide 1	IDLC_HUMAN	O14645	29.9	1	4.7	1	
Calicin	CALI_HUMAN	Q13939	67.539	1	2.2	1	
Catenin alpha-2	CTNA2_HUMAN	P26232	105.9	3	3.4	1	
Ciliary dynein heavy chain 9	DYH9_HUMAN	Q9NYC9	515.6	1	0.3	1	
Cytoskeleton-associated protein 2	CKAP2_HUMAN	Q8WWK9	77.5	2	2.8	1	
Desmoplakin	DESP_HUMAN	P15924	331.8	2	0.7	1	
Dynein intermediate chain 1, axonemal	DNAI1_HUMAN	Q9UI46	79.7	1	1.7	1	
Gelsolin precursor	GELS_HUMAN	P06396	86.0	1	1.7	1	
Hornerin	HORN_HUMAN	Q86YZ3	283.1	3	1.6	3	
Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	MACF1_HUMAN	Q9UPN3	623.6	5	1.0	1	
Microtubule-actin cross linking factor 1, isoform 4	MACF4_HUMAN	Q96PK2	673.7	4	1.0	2	
Myosin heavy chain, cardiac muscle beta isoform	MYH7_HUMAN	P12883	223.8	2	1.5	1	
Periplakin	PEPL_HUMAN	O60437	205.1	4	2.2	1	
Plectin-1	PLEC1_HUMAN	Q15149	531.7	3	0.9	1	
Protein piccolo	PCLO_HUMAN	Q9Y6V0	568.3	5	1.2	1	
Rootletin	CROCC_HUMAN	Q5TZA2	228.8	2	0.9	1	
Smoothelin	SMOO_HUMAN	P53814	100.0	3	3.2	3	
Titin (Connectin)	TITIN_HUMAN	Q8WZ42	3843.6	7	0.3	1	
Tubulin alpha-2 chain	TBA2_HUMAN	Q13748	50.6	9	26.7	3	[28, 40, 98]
Tubulin alpha-3 chain	TBA3_HUMAN	Q71U36	50.8	7	20.8	1	[28, 40, 98]
Tubulin alpha-ubiquitous chain	TBAK_HUMAN	P68363	50.8	5	13.5	4	[28, 40, 98]
Tubulin beta-1 chain	TBB1_HUMAN	Q9H4B7	50.9	2	6.9	1	[28, 40]
Tubulin beta-2C chain	TBB2C_HUMAN	P68371	50.3	12	32.4	5	[28, 40]
Tubulin beta-3 chain	TBB3_HUMAN	Q13509	50.9	9	23.1	4	[28, 40]
Tubulin beta-4q chain	TBB4Q_HUMAN	Q99867	48.9	4	11.1	1	[28, 40, 99, 100]
Tubulin beta-6 chain	TBB6_HUMAN	Q9BUF5	50.3	6	18.2	2	[28, 40]
Stress-Related Proteins							

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Chaperonin containing TCP1, subunit 3 (gamma) variant		BAD92119.1	61.1	4	7.8	2	
Chaperonin containing TCP1, subunit 8	NP_006576.2		60.0	3	6.9	2	
Endoplasmin precursor	ENPL_HUMAN	P14625	92.696	4	4.9	2	[101]
78 kDa glucose-regulated protein precursor	GRP78_HUMAN	P11021	72.4	2	3.7	1	[101, 102]
Glutathione S-transferase A3	GSTA3_HUMAN	Q16772	25.3	2	10.8	1	
Glutathione S-transferase Mu 2	GSTM2_HUMAN	P28161	25.8	1	5.2	1	
Glutathione S-transferase Mu 3	GSTM3_HUMAN	P21266	26.9	7	37.3	4	
Heat shock 70 kDa protein 1	HSP71_HUMAN	P08107	71.082	5	8.1	1	
Heat shock 70 kDa protein 1L	HS70L_HUMAN	P34931	70.73	5	9.5	1	
Heat shock 70 kDa protein 6	HSP76_HUMAN	P17066	71.44	7	10.4	4	
Heat shock cognate 71 kDa protein	HSP7C_HUMAN	P11142	71.082	8	11.3	4	[97, 101]
Heat shock protein 60 kDa	CH60_HUMAN	P10809	61.187	3	7.2	2	[28, 97, 102, 103]
Heat shock protein 75 kDa	TRAP1_HUMAN	Q12931	80.345	1	2.3	2	
Heat shock protein HSP 90-alpha	HS90A_HUMAN	P07900	84.875	23	25.4	4	[40, 99, 100, 103-105]
Heat shock protein HSP 90-beta	HS90B_HUMAN	P08238	83.423	12	18.5	2	[40, 99, 100, 103-105]
Heat shock-related 70 kDa protein 2	HSP72_HUMAN	P54652	70.263	22	36.2	4	
2-hydroxyacyl-CoA lyase 1	HACL1_HUMAN	Q9UJ83	64.4	2	3.6	1	
KIAA0098		BAA07894.2	60.1	4	8.3	2	
Peroxisome biogenesis factor 1	PEX1_HUMAN	O43933	143.8	2	2.0	4	
Stress-70 protein, mitochondrial precursor	GRP75_HUMAN	P38646	73.9	5	9.3	1	[27, 102]
T-complex 1		AAP36354.1	60.8	1	2.2	1	[103]
T-complex protein 1 subunit zeta-2	TCPW_HUMAN	Q92526	58.2	1	2.1	1	[27]
		Metabolic Enzymes					
Acetyl-CoA acetyltransferase, mitochondrial precursor	THIL_HUMAN	P24752	45.5	6	15.9	1	[102]
Adenylate kinase isoenzyme 1	KAD1_HUMAN	P00568	21.7	3	16.0	2	
Alpha-enolase	ENOA_HUMAN	P06733	47.3	10	31.6	3	[30, 97-101, 103, 105]

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Aspartate aminotransferase, cytoplasmic	AATC_HUMAN	P17174	46.3	2	6.5	1	
Aspartate aminotransferase, mitochondrial precursor	AATM_HUMAN	P00505	47.8	1	2.6	1	[26]
3′(2′), 5′-biphosphate nucleotidase 1	BPNT1_HUMAN	O95861	33.7	1	3.3	1	
Carbonic anhydrase 2	CAH2_HUMAN	P00918	29.154	1	4.2	1	[100, 106, 107]
Carnitine O-acetyltransferase	CACP_HUMAN	P43155	71.3	3	4.8	1	
11-cis retinol dehydrogenase	RDH1_HUMAN	Q92781	35.3	1	4.4	1	
Citrate synthase, mitochondrial precursor	CISY_HUMAN	O75390	51.9	6	15.0	3	
Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial precursor	ECH1_HUMAN	Q13011	36.1	3	9.2	1	
2,4-dienoyl-CoA reductase, mitochondrial precursor	DECR_HUMAN	Q16698	36.3	5	16.1	1	[102]
Dihydrolipoyl dehydrogenase, mitochondrial precursor	DLDH_HUMAN	P09622	54.7	3	8.3	1	[26]
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor	ODP2_HUMAN	P10515	66.2	2	3.6	2	
Dihydroxyacetone kinase	DAK_HUMAN	Q3LXA3	59.3	2	3.5	1	
Ethanolamine-phosphate cytiddylyltransferase	PCY2_HUMAN	Q99447	44.3	1	4.1	1	
Fructose-bisphosphate aldolase A	ALDOA_HUMAN	P04075	39.7	11	34.3	3	[99, 100, 103, 105, 108]
Fumarate hydratase, mitochondrial precursor	FUMH_HUMAN	P07954	54.8	6	14.1	1	
Gamma-glutamyl hydrolase precursor	GGH_HUMAN	Q92820	36.3	2	9.8	1	
Glucose-6-phosphate isomerase	G6PI_HUMAN	P06744	63.2	6	44.2	2	
Glutamine-dependent NAD (+) synthetase	NADE1_HUMAN	Q6IA69	80.5	1	1.6	1	
Glutamine synthetase	GLNA_HUMAN	P15104	42.5	4	11.1	2	[30]
Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	P04406	36.1	1	3.6	2	[28, 40, 41, 91, 92, 97, 98, 105, 107, 109-114]

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Hexokinase-1	HXK1_HUMAN	P19367	103.6	16	18.3	2	[40, 92]
Histidine ammonia-lyase	HUTH_HUMAN	P42357	73.3	1	1.6	1	
3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor	3HIDH_HUMAN	P31937	35.7	1	4.5	1	[102]
Kinesin family member 21B	KI21B_HUMAN	O75037	184.3	3	2.3	1	
Lactotransferrin	TRFL_HUMAN	P02788	80.0	8	13.2	2	
L-lactate dehydrogenase A chain	LDHA_HUMAN	P00338	36.8	7	27.1	3	
L-Lactate dehydrogenase B chain	LDHB_HUMAN	P07195	36.8	4	14.7	3	[101]
L-lactate dehydrogenase A-like 6A	LDH6A_HUMAN	Q6ZMR3	36.8	2	7.2	1	
L-lactate dehydrogenase A-like 6B	LDH6B_HUMAN	Q9BYZ2	42.4	3	8.7	2	
Long-chain-fatty-acid-CoA ligase 6	ACSL6_HUMAN	Q9UKU0	78.8	1	1.9	1	
Malate dehydrogenase, cytoplasmic	MDHC_HUMAN	P40925	36.5	1	3.3	1	[28, 97, 100, 115]
Malate dehydrogenase, mitochondrial precursor	MDHM_HUMAN	P40926	36.0	13	43.8	3	[26, 100, 102]
Mitochondrial dicarboxylate carrier	DIC_HUMAN	Q9UBX3	31.7	1	3.5	1	
NAD-dependent malic enzyme, mitochondrial precursor	MAOM_HUMAN	P23368	66.0	2	1.5	1	
6-phosphofructokinase type C	K6PP_HUMAN	Q01813	86.4	11	13.5	2	[28]
Phosphoglycerate kinase 1	PGK1_HUMAN	P00558	44.8	2	4.6	1	[100, 103]
Phosphoglycerate mutase 1	PGAM1_HUMAN	P18669	28.8	1	4.7	1	
Phosphoglycerate mutase 2	PGAM2_HUMAN	P15259	28.8	4	18.6	2	
Probable saccharopine dehydrogenase	SCPDH_HUMAN	Q8NBX0	47.5	7	20.8	2	
Protein disulfide-isomerase A3 precursor	PDIA3_HUMAN	P30101	57.1	2	5.5	1	[97, 102, 103]
Pyruvate dehydrogenase E1 component alpha subunit, testis specific form, mitochondrial precursor	ODPAT_HUMAN	P29803	43.6	3	8.5	1	
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial precursor	ODPB_HUMAN	P11177	39.5	2	6.4	1	

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Pyruvate kinase isozymes M1/M2	KPYM_HUMAN	P14618	58.3	14	29.2	3	[28, 98]
Transaldolase	TALDO_HUMAN	P37837	37.7	2	6.8	1	
Triosephosphate isomerase	TPIS_HUMAN	P60174	26.8	9	44.2	3	[98-100, 103, 105, 108]
Cell Cycle							
Apoptosis-stimulating of p53 protein 2	ASPP2_HUMAN	Q13625	126.2	1	0.9	3	
Histone H1.3	H13_HUMAN	P16402	22.2	2	10.0	1	
Microtubule-associated protein 1A	MAP1A_HUMAN	P78559	307.6	1	0.3	1	
Nicotinamide phosphoribosyltransferase	NAMPT_HUMAN	P43490	55.8	1	2.7	1	
Peroxiredoxin-1	PRDX1_HUMAN	Q06830	22.3	1	6.0	1	[91, 103]
Translin	TSN_HUMAN	Q15631	26.3	2	9.7	2	
Transcription Factors							
Centromere protein F	CENPF_HUMAN	P49454	357.4	2	0.8	1	
HIV Tat-specific factor 1	HTSF1_HUMAN	O43719	86.4	3	4.4	2	
Protein bassoon	BSN_HUMAN	Q9UPA5	418.2	6	1.6	2	[28]
SW1/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily C member 1	SMRC1_HUMAN	Q92922	123.2	2	2.4	1	
Zinc finger protein 395	ZN395_HUMAN	Q9H8N7	55.7	1	1.6	1	
Zinc finger protein 512	ZN512_HUMAN	Q96ME7	65.7	3	5.6	1	
Zinc finger protein 696	ZN696_HUMAN	Q9H7X3	41.7	2	5.9	1	
Zinc finger protein 740	ZN740_HUMAN	Q8NDX6	22.5	1	5.7	1	
Protein Binding							
Ankyrin-3	ANK3_HUMAN	Q12955	482.4	5	1.4	1	
Ankyrin repeat domain-containing protein 11	ANR11_HUMAN	Q6UB99	299.8	4	1.6	2	
Calcium-binding tyrosine phosphorylation-regulated protein	CABYR_HUMAN	O75952	52.9	10	19.7	4	
Coiled-coil alpha-helical rod protein 1	CCHCR_HUMAN	Q8TD31	88.9	1	1.0	1	
Coiled-coil domain-containing	CCD71_HUMAN	Q8IV32	49.8	3	6.4	2	

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
protein 71							
Collagen alpha-2 (VI) chain precursor	CO6A2_HUMAN	P12110	109.7	2	2.2	1	
Collagen alpha-3 (IV) chain precursor	CO4A3_HUMAN	Q01955	163.0	2	1.7	1	
Cullin-3	CUL3_HUMAN	Q13618	89.4	3	4.4	3	
Cytoskeleton-associated protein 5	CKAP5_HUMAN	Q14008	225.5	2	0.9	1	
DNA replication licensing factor MCM6	MCM6_HUMAN	Q14566	93.8	4	5.9	1	
Elongation factor 1-gamma	EF1G_HUMAN	P26641	50.3	4	10.2	1	
Elongation factor 2	EF2_HUMAN	P13639	96.1	5	6.1	1	[27, 98, 100, 101, 103, 105]
Extracellular matrix protein 2 precursor	ECM2_HUMAN	O94769	80.6	3	5.2	1	
Liprin-alpha-1	LIPA1_HUMAN	Q13136	136.3	2	1.7	1	
Neurexin-1-alpha precursor	NRX1A_HUMAN	Q9ULB1	164.1	3	2.2	1	
Plasminogen		AAH60513.1	93.3	1	1.5	2	[95, 116]
Ran-binding protein 2-like 3	RBP23_HUMAN	O14715	84.9	3	4.9	1	
RuvB-like 1	RUVB1_HUMAN	Q9Y265	50.5	3	8.6	1	
RuvB-like 2	RUVB2_HUMAN	Q9Y230	51.2	4	10.2	1	
Tankyrase-1	TNKS1_HUMAN	O95271	143.7	3	1.7	1	
Transitional endoplasmic reticulum ATPase	TERA_HUMAN	AAC07984.1	89.8	5	7.7	1	[100]
Vang-like protein 2	VANG2_HUMAN	Q9ULK5	59.9	2	4.2	1	
Protein turnover							
Cytosol aminopeptidase	AMPL_HUMAN	P28838	53.0	2	5.5	2	
E3 Ubiquitin-protein ligase UBR1	UBR1_HUMAN	Q8IWV7	203.4	2	1.0	1	
Matrix metalloproteinase-24 precursor	MMP24_HUMAN	Q9Y5R2	73.6	1	1.9	1	
Probable aminopeptidase NPEPL1	PEPL1_HUMAN	Q8NDH3	56.7	1	2.7	1	
Probable serine carboxypeptidase CPVL precursor	CPVL_HUMAN	Q9H3G5	54.4	2	5.9	1	
26S protease regulatory subunit 4	PRS4_HUMAN	P62191	49.3	2	5.7	1	

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Proteasome subunit alpha type 2	PSA2_HUMAN	P25787	25.9	1	5.6	2	
Proteasome subunit alpha type 3	PSA3_HUMAN	P25788	28.5	1	5.5	1	
Proteasome subunit alpha type 5	PSA5_HUMAN	P28066	26.6	2	9.5	1	
Proteasome subunit alpha type 6	PSA6_HUMAN	P60900	27.8	1	6.1	1	
Proteasome subunit alpha type 7-like	PSA7L_HUMAN	Q8TAA3	28.7	2	11.3	1	
Proteasome subunit beta type 3	PSB3_HUMAN	P49720	23.2	1	5.4	1	
Proteasome subunit beta type 5 precursor	PSB5_HUMAN	P28074	23.0	1	7.2	1	
Proteasome subunit beta type 6 precursor	PSB6_HUMAN	P28072	25.6	3	9.6	2	
Serine protease inhibitor Kazal-type 5 precursor	ISK5_HUMAN	Q9NQ38	124.4	2	2.1	1	
Energy							
ATP synthase subunit alpha, mitochondrial precursor	ATPA_HUMAN	P25705	59.8	5	10.1	2	[30, 100, 102, 117]
ATP synthase B Chain, mitochondrial precursor	AT5F1_HUMAN	P24539	28.9	1	3.9	1	[100, 102]
ATP synthase gamma chain, mitochondrial precursor	ATPG_HUMAN	P36542	33.0	2	8.4	2	
ATP synthase O subunit, mitochondrial precursor	ATPO_HUMAN	P48047	23.4	2	12.6	1	
Electron transfer flavoprotein subunit beta	ETFB_HUMAN	P38117	27.9	6	25.9	2	[102]
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor	DHSA_HUMAN	P31040	73.7	3	6.2	2	[102]
Trifunctoinal enzyme subunit alpha, mitochondrial precursor	ECHA_HUMAN	P40939	83.7	5	7.2	1	
Trifunctional enzyme subunit beta, mitochondrial precursor	ECHB_HUMAN	P55084	51.5	3	7.2	1	
4-trimethylaminobutyraldehyde dehydrogenase	AL9A1_HUMAN	P49189	54.7	1	2.6	1	[92, 118]
Testis/Sperm Proteins							
Acrosin-binding protein precursor	ACRBP_HUMAN	Q8NEB7	62.4	5	9.8	3	
Acrosin precursor	ACRO_HUMAN	P10323	25.5	4	11.9	3	

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Glyceraldehyde-3-phosphate dehydrogenase, testis specific	G3PT_HUMAN	O14556	44.5	11	36.3	4	
L-lactate dehydrogenase C chain	LDHC_HUMAN	P07864	36.5	17	53.0	4	
Nucleoside diphosphate kinase homolog 5	NDK5_HUMAN	P56597	24.4	1	4.7	1	
Outer dense fiber of sperm tails 2 isoform 3		AAP83847.1	76.1	5	8.8	1	
Outer dense fiber protein	ODFP_HUMAN	Q14990	30.4	3	11.2	2	
Phosphoglycerate kinase, testis specific	PGK2_HUMAN	P07205	45.0	10	29.5	3	
RANBP2-like and GRIP domain-containing protein 8	RGPD8_HUMAN	Q99666	200.0	3	1.8	1	
Semenogelin-1 precursor	SEMG1_HUMAN	P04279	52.2	3	9.1	2	
Semenogelin-2 precursor	SEMG2_HUMAN	Q02383	65.5	4	8.6	2	
Sperm protein associated with the nucleus on the X chromosome A	SPNXA_HUMAN	Q9NS26	11.1	1	13.4	2	
Sperm protein associated with the nucleus on the X chromosome B/F	SPNXB_HUMAN	Q9NS25	11.9	3	47.6	3	
Testis-specific Y-encoded-like protein 3	TSYL3_HUMAN	Q9H489	39.8	2	4.5	1	
Zona pellucida-binding protein 1	ZPBP1_HUMAN	Q9BS86	41.0	5	12.5	3	
Others							
Dermcidin precursor	DCD_HUMAN	P81605	11.4	2	22.7	1	
Disks large-associated protein 3	DLGP3_HUMAN	O95886	106.9	1	1.0	1	
DNA (cytosine-5)-methyltransferase 1	DNMT1_HUMAN	P26358	185.4	3	2.3	1	[92, 119, 120]
DnaJ homolog subfamily B member 6	DNJB6_HUMAN	O75190	36.122	3	9.5	2	
ES1 protein homolog, mitochondrial precursor	ES1_HUMAN	P30042	28.5	3	13.1	2	
FERM domain-containing protein 4A	FRM4A_HUMAN	Q9P2Q2	114.5	3	3.2	1	
GRAM domain-containing protein 3	GRAM3_HUMAN	Q96HH9	48.3	2	5.1	1	
Hemicentin-1 precursor	HMCN1_HUMAN	Q96RW7	62.3	4	1.0	1	

Europe PMC Funders Author Manuscripts

Protein Name	Swiss-Prot		Mol. mass (kDa)	No. of Unique Peptides	Sequence Coverage (%)	No. of Exp.[c]	Previously Identified as S-Nitrosylated Targets
	Entry Name	Accession No.					
Kelch-like protein 10	KLH10_HUMAN	Q6JEL2	69.9	2	3.6	1	
Leucine-rich repeat-containing protein 37A	LR37A_HUMAN	O60309	181.6	1	0.6	1	
Leucine-rich repeat-containing protein 37B precursor	LR37B_HUMAN	Q96QE4	106.4	6	8.3	3	
Myeloid leukaemia factor 1	MLF1_HUMAN	P58340	30.7	3	11.9	2	
Myeloid/lymphoid or mixed-lineage leukaemia protein 3 homolog	MLL3_HUMAN	Q8NEZ4	548.2	5	1.0	2	
Nesprin-2	SYNE2_HUMAN	Q8WXH0	801.8	3	0.5	1	
Periaxin	PRAX_HUMAN	Q9BXM0	155.2	2	1.4	1	
Poly [ADP-ribose] polymerase 14	PAR14_HUMAN	Q460N5	195.6	3	1.6	1	
Prostate-specific antigen precursor	KLK3_HUMAN	P07288	29.3	2	8.8	1	
Protein FAM29A	FA29A_HUMAN	Q7Z4H7	109.6	2	2.5	1	
Regulating synaptic membrane exocytosis protein 1	RIMS1_HUMAN	Q86UR5	189.0	4	2.5	3	
Scaffold attachment factor B	SAFB1_HUMAN	Q15424	103.0	3	3.7	1	
SH3 domain and tetratricopeptide repeats-containing protein 1	S3TC1_HUMAN	Q8TE82	148.5	2	2.0	2	
Spindle assembly abnormal protein 6 homolog	SAS6_HUMAN	Q6UVJ0	74.8	2	4.1	1	
Stathmin-2	STMN2_HUMAN	Q93045	20.9	2	11.7	1	
Targeting protein for Xklp2	TPX2_HUMAN	Q9ULW0	86.2	2	2.4	1	
Thioredoxin domain-containing protein 3	TXND3_HUMAN	Q8N427	67.7	3	6.6	1	
Thioredoxin domain-containing protein 11	TXD11_HUMAN	Q6PKC3	111.8	2	2.4	2	
Tripartite motif-containing protein 34	TRI34_HUMAN	Q9BYJ4	58.1	2	1.9	1	
Tryptophane aspartate-containing coat protein	AF495470_1	AAM18516	51.5	1	3.9	1	
Uncharacterized protein C6orf54	CF054_HUMAN	Q9Y6Z4	20.1	2	12.2	1	

[a] MOWSE scores >33

[b] See supplementary Table S1 for additional information.

[c] Number of experiment refers to how many times a particular protein was identified out of a total of 5 independent experiments.

Europe PMC Funders Author Manuscripts