Redox-dependent thiol modifications: implications for the release of extracellular vesicles

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Abstract
Extracellular vesicles (EVs), including microvesicles and exosomes, are emerging as important regulators of homeostasis and pathophysiology. During pro-inflammatory and pro-oxidant conditions, EV release is induced. As EVs released under such conditions often exert pro-inflammatory and procoagulant effects, they may actively promote the pathogenesis of chronic diseases. There is evidence that thiol group-containing antioxidants can prevent EV induction by pro-inflammatory and oxidative stimuli, likely by protecting protein thiols of the EV-secreting cells from oxidation. As the redox state of protein thiols greatly impacts three-dimensional protein structure and, consequently, function, redox modifications of protein thiols may directly modulate EV release in response to changes in the cell’s redox environment. In this review article, we discuss targets of redox-dependent thiol modifications that are known or expected to be involved in the regulation of EV release, namely redox-sensitive calcium channels, N-ethylmaleimide sensitive factor, protein disulfide isomerase, phospholipid flippases, actin filaments, calpains and cell surface-exposed thiols. Thiol protection is proposed as a strategy for preventing detrimental changes in EV signaling in response to inflammation and oxidative stress. Identification of the thiol-containing proteins that modulate EV release in pro-oxidant environments could provide a rationale for broad application of thiol group-containing antioxidants in chronic inflammatory diseases.

Keywords  Exosomes · Microvesicles · Sulphhydryl groups · Redox environment · Chronic inflammation · N-acetyl-l-cysteine

Abbreviations
AFF-1 Anchor cell fusion failure 1
AnxV Annexin V
ATP Adenosine triphosphate
CSE Cigarette smoke extract
DTNB 5,5-Dithio-bis-(2-nitrobenzoic acid)
EFF-1 Epithelial fusion failure 1
ER Endoplasmic reticulum
ESCRT Endosomal sorting complex required for transport
EV Extracellular vesicle
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GSH Glutathione
HCAEC Human coronary artery endothelial cells
HNE 4-Hydroxy-2-nonenal
ILV Intraluminal vesicle
LPS Lipopolysaccharide
MPG N-(2-Mercaptopropionyl)glycine
MVE Multivesicular endosome
NAC N-acetyl-l-cysteine
Extracellular vesicles

Extracellular vesicles (EVs) are small membrane-surrounded vesicles that are secreted by virtually all cell types and that have been detected in various body fluids including plasma [1], urine [2] and bronchoalveolar lavage fluid [3]. These EVs carry a complex cargo composed of proteins, nucleotides and lipids, among others [4]. They can exert multiple biological effects, either by interacting with or being taken up by target cells, or in the extracellular space [5]. As EVs are released under physiological and stress conditions, they have been attributed various functions in homeostasis as well as pathology [5].

Pathophysiological relevance of redox-mediated EV signaling

EVs are actively involved in the pathophysiology of conditions that are associated with local or systemic inflammation and oxidative stress, such as unhealthy aging (also termed ‘inflammaging’), cancer, cardiovascular disease (CVD) and chronic lung diseases [13–16]. Several in vitro studies have shown that exposure of EV-secreting cells to pro-inflammatory or pro-oxidant conditions causes pathological changes in EV signaling [17, 18]. Moreover, chronic inflammatory

Table 1 Summary of the differential properties of exosomes and microvesicles. ESCRT, endosomal sorting complex required for transport; MVB, multivesicular body

|                     | Exosomes                      | Microvesicles                      |
|---------------------|-------------------------------|-----------------------------------|
| Alternative names   | Extracellular vesicles        | Extracellular vesicles, microparticles, shedding vesicles, ectosomes |
| Size                | 50–150 nm                     | 100–1000 nm                       |
| Biogenesis          | As ILVs in MVBs, followed by extracellular release by fusion of the MVBs with the plasma membrane | Direct shedding from the plasma membrane |
| Characteristic proteins | Tetraspanins (e.g., CD63, CD81, CD9), ESCRT components (ALIX, TSG101) | Integrins, selectins |
diseases are associated with oxidative stress as well as elevated EV concentrations and altered EV composition, both of which can contribute to adverse biological effects of EVs [15, 19, 20]. Pharmacological modulation of EV formation may, therefore, be a promising treatment strategy for multiple chronic inflammatory conditions. However, most known targets for inhibiting EV release, including neutral sphingomyelinase 2 and the GTPase Rab27a [21, 22], are required for vital cellular processes such as lipid biosynthesis and intracellular membrane trafficking [23, 24]. Therefore, currently known strategies for EV inhibition are likely associated with off-target effects, hampering their application for the therapeutic modulation of EV signaling in vivo. An improved understanding of the mechanisms that modulate EV release under conditions of chronic inflammation and oxidative stress could allow identifying pharmacological compounds that only prevent pathological changes in EV signaling, but do not interfere with the physiological functions of EVs. Protein thiols can undergo reversible redox modifications. Thereby, they can act as switches in cellular redox signaling, and determine the cell’s response to changes in its redox environment [25, 26]. In this review article, we discuss how thiol modifications may be involved in EV formation and propose thiol protection as a novel strategy for preventing pathological changes in EV signaling.

Redox regulation of protein thiols

This section provides general background information on how redox modifications of protein thiols affect protein function and, consequently, cellular functions. It aims to familiarize the reader with important principles and terminology in the field of redox-dependent thiol modifications before we discuss “Modulation of EV signaling by thiol modifications” in the next section.

Thiol groups in proteins are contributed by the amino acid cysteine. Most commonly, protein thiols exist either in a free, reduced form (Cys–SH), or oxidized to a disulfide with another thiol group of the same or another protein (Cys–S–S–Cys) [27]. During protein biosynthesis, disulfide bonds are introduced in a highly regulated manner. They are essential for correct three-dimensional protein structure and, consequently, for protein function. To assure correct protein folding, the position of cysteine residues within proteins is highly conserved [28]. Reduced thiols are nucleophilic and susceptible to electrophilic attacks because of their electron-rich sulfur atom [25]. Certain protein microenvironments polarize the S–H bond and thereby lower the acid dissociation constant (pKₐ) of thiol groups. This favors their deprotonation (i.e., the dissociation of H⁺), resulting in the formation of even more reactive thiolate anions (Cys–S⁻) [27]. This often occurs at active site cysteines, making them especially susceptible for redox modifications [29].

Electrophilic compounds that are prone to react with thiolates or thiols include reactive oxygen species (ROS), which can lead to the formation of ectopic disulfide bonds, reactive nitrogen species (RNS) which lead to S-nitrosylation or S-nitration (Cys-S-NO) and reactive carbonyl species (RCS) which result in adduct formation (Cys-S-R) [26] (illustrated in Fig. 1). ROS, RNS and RCS are present in environmental exposures, including cigarette smoke and vehicle emissions [30–32] and are also formed endogenously, for instance, during inflammation and lipid peroxidation [33–35]. Reaction of such oxidative stimuli with protein thiols can strongly affect protein conformation and functionality and cysteine-rich proteins often undergo redox regulation, especially those containing unpaired cysteines with free thiol groups [27]. The cell has a powerful antioxidant system at its disposal, which consists of glutathione and various antioxidant enzymes [36]. Up to moderate oxidant levels, this system is able to control and reverse thiol modifications by ROS and RCS, thereby allowing oxidative thiol modifications to act as transient and specific cell signaling events [36, 37]. Studies that investigate the regulation of protein function by redox sensitive thiols often apply electrophilic compounds that covalently modify thiols, trapping them either transiently or irreversibly in an oxidized state [29]. Here, we will refer to these chemicals as thiol scavengers.

In the following sections, we discuss how thiol modifications may be involved in EV formation and how thiol protection may be used to prevent pathological changes in EV signaling.

Modulation of EV signaling by thiol modifications

Modulation of EV release

Intriguingly, it was reported as early as 1961 that treatment of various cells with thiol-reactive compounds induces blebbing of the plasma membrane [38]. In 1979, Scott and Maerccklein found that these blebs were released into the cell culture media as 100 nm- to 10 µm-sized vesicles and could be isolated by centrifugation at 30,000xg [39]. The phenomenon was universal for cultured cells from different species and tissues, including fibroblasts, monocytes and macrophages and could be induced by a wide panel of RCS, including formaldehyde, N-ethyl-maleimide (NEM) and acrolein [39]. More recently, we and others have confirmed that treatment of various cell types with RCS or ROS enhances the release of EVs (for details, see Table 2) [40–43]. A number of studies have shown that both ROS-dependent and RCS-dependent EV induction are preventable by thiol-based
antioxidants such as NAC [41–43], suggesting that thiol-reactivity is causally linked to EV induction. Yet, while Vatsyayan et al. proposed that RCS-induced EV release is mediated by secondary ROS generation, we found that only RCS, but not the ROS hydrogen peroxide, elicit increased EV release [40, 41]. Vatsyayan et al. and the studies that showed ROS-induced EV release detected EVs by direct flow cytometry for microvesicles (detection limit 300 nm) [40, 42, 43], whereas we used a combination of tunable resistive pulse sensing and bead-based flow cytometry to detect small EVs (85–250 nm) expressing exosome marker proteins [41]. Therefore, thiol modifications by RCS and ROS may differentially affect the release of microvesicles and exosomes. Moreover, it may depend on the cell type and its repertoire of proteins with redox sensitive thiols whether a certain stimulus does or does not trigger EV release. For instance, in the study by Vatsyayan et al., cell exposure to the RCS 4-hydroxy-2-nonenal enhanced the release of EVs by endothelial cells and fibroblasts, but not by monocytes [40].

In some studies, ROS or RCS-induced microvesicle production was associated with a considerable amount of apoptotic cell death [23, 48], suggesting that microvesicle shedding in the response to thiol-reactive compounds may be due to cytotoxicity rather than being directly mediated by thiol modifications. However, we have found that the thiol scavengers 5,5-dithio-bis-(2-nitrobenzoic acid) DTNB and bacitracin induce EVs without affecting cell viability [29] and other mechanisms have been implied in ROS and RCS-dependent EV induction, as will be discussed later. While the effect of RNS on EV release is less well studied than that of ROS and RCS, there is evidence that nitric oxide (NO) negatively regulates EV release [44, 45]. Table 2 gives an overview of the experimental evidence for modulation of EV release by thiol-reactive compounds.

**Modulation of EV cargo and functions**

Functional implications of EVs released under oxidative stress conditions have been described in detail elsewhere [18, 46]. Therefore, we will only discuss those studies that explicitly investigated EV functions related to oxidative thiol modifications. Szabó-Taylor et al. have exposed monocytes to pro-inflammatory conditions associated with oxidative stress and assessed the expression of the thiol-dependent redox enzyme peroxiredoxin 1 on the cells and their EVs [47]. While exofacial peroxiredoxin 1 was readily detectable on both, secreting cells and EVs, the over oxidized and enzymatically inactive form was exclusively enriched on the EVs [47]. This suggests that cells may release membrane proteins with oxidized thiols on EVs to maintain a reduced membrane status in oxidative environments. Similarly, the thiol groups of the cytosolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) become oxidized during red blood cell storage and the oxidized form of GAPDH is then released in an EV-associated manner [48]. This implies that intraluminal proteins with oxidized thiols may also be released in EVs as a protective mechanism.
Table 2: Experimental evidence for the involvement of protein thiols in the regulation of EV release

| Study                        | Cell type                              | Exogenous thiol-reactive compounds                                                                 | Exposure time | Endogenous thiol-reactive compounds | Thiol antioxidants | EV nomenclature | Effect of the thiol-reactive compounds on EV release | Summary of major findings                                                                 |
|------------------------------|----------------------------------------|--------------------------------------------------------------------------------------------------|---------------|-------------------------------------|-------------------|-----------------|-----------------------------------------------------|--------------------------------------------------------------------------------------------|
| Belkin and Hardy [38]        | Ascites cells, malignant cells, non-malignant cells | RCS (NEM; iodoacetamide)\[ ROS (H₂O₂)\] Others (mercurial diuretics)                                       | ≤ 60 min      | n.d.                                | None              | Plasma membrane blebs | ↑                                                   | All tested cell types respond to treatment with various extrinsic thiol-reactive compounds by plasma membrane blebbing |
| Scott et al. [39]            | Fibroblasts, monocytes, myoblasts, etc. | RCS (e.g., formaldehyde, acrolein, NEM, iodoacetate)                                                                 | 30 min        | n.d.                                | None              | Plasma membrane blebs/vesicles | ↑                                                   | All tested RCS, but not control compounds that do not react with thiols (e.g., succinimide), induce plasma membrane blebbing |
| Dachary-Prigent et al. [97]  | Human platelets                        | RCS (NEM, diamide)                                                                                                                                     | 10 min        | n.d.                                | None              | Microparticles   | (↓)                                                | Platelet preincubation with RCS inhibits ionophore-induced calpain activity, PS externalization and EV release |
| Furlan-Freguia et al. [52]   | Murine macrophages and smooth muscle cells | Thiol scavenger (DTNB)                                                                              | 30 min        | ROS                                 | NAC               | Microparticles   | ↑                                                   | ATP stimulation results in endogenous ROS formation and release of procoagulant EVs. Blocking cell surface thiols with DTNB and ROS scavenging both prevent ATP-induced EV release |
| Vatsyayan et al. [40]        | Human macrophages, coronary artery endothelial cells, fibroblasts | RCS (HNE, acrolein)                                                                                                                                  | 15 min–4 h    | ROS                                 | NAC, MPG          | Microparticles   | ↑                                                   | In endothelial cells and fibroblasts, but not monocytes, extrinsic RCS induce procoagulant EVs. Intrinsic ROS generation and PS externalization are increased in all cell types and preventable by thiol protection |
| Study                  | Cell type                                      | Exogenous thiol-reactive compounds | Exposure time | Endogenous thiol-reactive compounds | Thiol antioxidants | EV nomenclature | Effect of the thiol-reactive compounds on EV release | Summary of major findings |
|-----------------------|------------------------------------------------|-----------------------------------|---------------|-------------------------------------|--------------------|-----------------|------------------------------------------------------|---------------------------|
| Novelli et al. [42]   | Human alveolar and bronchial epithelial cells  | ROS (H$_2$O$_2$)                  | 20 h          | n.d.                                | NAC                | Microparticles  | ↑                                                    | Extrinsic ROS induce the release of pro-coagulant EVs by alveolar and bronchial epithelial cells, which is prevented by thiol protection |
| Carver et al. [43]    | Human retinal pigment epithelial cells         | ROS (H$_2$O$_2$)                  | 2–24 h        | n.d.                                | NACA               | Microparticles  | ↑                                                    | Extrinsic ROS induce EV release. The EV release correlates with cellular apoptosis and is preventable by thiol protection |
| Szabó-Taylor et al. [47] | Human monocytes                              | None                              | 90 min        | n.d.                                | None               | Extracellular vesicles | Not determined                  | Monocytes from pro-inflammatory conditions have increased exofacial thiols, but release EVs with decreased exofacial thiols. These EVs carry overoxidized proteins |
| Thom et al. [67]      | Human and murine neutrophils and monocytes    | None                              | 2 h           | ROS/RNS                             | None               | Microparticles  | ↑                                                    | Treatment of neutrophils with CO$_2$ activates mitochondrial ROS generation and subsequent thiol-dependent activation of IP3 receptors. This causes calcium flux from the ER to the cytoplasm, and S-nitrosylation of actin, resulting in increased EV release |
However, the release of EVs under thiol-depleting conditions may not only confer cellular protection, as EV-associated oxidized proteins and phospholipids can serve as danger-associated molecular patterns and trigger inflammation [49]. Oxidative thiol modifications also appear to promote coagulation in an EV-dependent manner. A variety of thiol-depleting oxidative and pro-inflammatory conditions have been found to result in accumulation of prothrombotic EVs in vitro and in vivo [40, 42, 50–53]. The prothrombotic effect of these EVs has been ascribed to the phospholipid phosphatidylserine (PS) [42, 50, 53] and to EV-associated tissue factor (TF) [40, 42, 50, 52]. PS-rich membranes provide a negatively charged surface for the assembly of coagulation factors and thereby promote coagulation [54]. Since PS is considered a universal constituent of the outer leaflet of EV membranes [55], an increase in the number of secreted EVs should be sufficient to enhance PS-dependent coagulation. TF is the initiator of the extrinsic coagulation cascade [56]. Expression and activity of TF are both increased in EVs secreted by cells stimulated with thiol-reactive compounds [40, 42, 50, 52]. Intriguingly, the activity of TF increases when its free thiol groups are oxidized to form a disulfide bond [57], suggesting that redox modifications are important regulators of both, quantity and activity of EV-associated TF.

**Summary**

Cell exposure to thiol-reactive compounds, particularly RCS and ROS, results in plasma membrane blebbing and increased release of EVs. EVs released in response to thiol modifications protect the secreting cell from oxidative damage, but also promote potentially harmful processes such as inflammation and coagulation.

**Molecular targets of thiol modifications that regulate EV release or EV uptake**

Although thiol modifications modulate the release and biological functions of EVs, relatively little is known about the thiol-bearing proteins that mediate these changes. In this section, various thiol-dependent mechanisms are presented that regulate either membrane fusion or blebbing and that are known or hypothesized to modulate EV release. These mechanisms are summarized in Table 3 and visualized in Fig. 2. Additionally, three recent publications are discussed in detail, which specifically imply modifications of cell surface-exposed thiols in EV release [41, 47, 52].
Redox sensitive calcium channels

Cytoplasmic calcium influx is a major inducer of both, microvesicle and exosome release, as it promotes membrane blebbing as well as fusion of MVBs with the plasma membrane [58, 59]. Intriguingly, several calcium channels bear redox-sensitive thiol groups and become activated upon their oxidation [60–64] (Fig. 2a; for details of the different calcium channels see Table 3). The nociceptor transient receptor potential ankyrin subtype 1 (TRPA1) is among the best studied calcium channels whose activity is modulated by thiol modifications [65]. Expressed by sensory neurons and other sensory cells, including epithelial cells [65], TRPA1 becomes activated upon covalent thiol oxidation by RCS or ROS [61, 66]. While a causal link between TRPA1 activation and induction of EV release has to our knowledge not been investigated, oxidative stimuli such as cigarette smoke and acrolein cause TRPA1 activation [63] and also enhance EV release [41]. Direct evidence that thiol-dependent calcium flux to the cytoplasm is associated with increased EV release stems from a study by Thom et al. The authors have shown that, ROS-dependent thiol oxidation of inositol-1,3,5-triphosphate (IP3) receptors triggers calcium flux from the endoplasmic reticulum to the cytoplasm and, consequently, EV release [67]. However, it should be noted that some calcium channels are inhibited, rather than activated by oxidative modification of their thiols [60]. Therefore, it may depend on the types of calcium channels expressed by a cell whether cytoplasmic calcium influx and, consequently, EV release is promoted or inhibited by cell exposure to thiol-reactive species.

Additionally to the direct effects of thiol modifications on EV release, the coming sections will also address how cytoplasmic calcium concentrations influence EV release.

SNAREs and NSF

Soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) mediate various intracellular membrane fusion events and are also involved in EV release [68–70]. In the presence of cytoplasmic calcium, e.g., upon activation of thiol-regulated calcium channels, vesicle-associated (v)-SNAREs and target membrane-associated (t)-SNAREs form a highly stable complex that can force two membranes together, resulting in membrane fusion (Fig. 2b) [71]. After SNARE-mediated fusion, the ATPase N-ethylmaleimide-sensitive factor (NSF) can translate the energy from ATP hydrolysis into a large conformational change that mechanically separates v-SNAREs and t-SNAREs, making them available for further membrane fusion events [71] (Fig. 2c). Importantly, activity of NSF depends on reduced thiols and can be inhibited by the thiol-reactive carbonyl N-ethyl-maleimide (NEM) [72]. SNAREs as well as NSF have been implicated in the fusion of MVBs with the plasma membrane, resulting in exosome release [69, 73, 74]. Taken together, under oxidative conditions, thiol-dependent calcium influx is expected to cause SNARE-dependent exosome release. Yet, oxidation of the free thiol group of NSF may prevent recovery of the SNARE proteins for subsequent membrane fusion events.

The protein disulfide isomerase family and thiol-rich fusion proteins

The protein disulfide isomerase (PDI) family is a family of proteins with thiol-dependent oxidoreductase activity. The prototype family member PDI is expressed abundantly in most tissues and has two thioredoxin-like active sites (-Cys-[X]-Cys-) [75]. Both active sites contain two cysteine residues, which catalyze various redox reactions, such as reduction of disulfide bonds, isomerization of disulfide bonds and oxidation of free thiols, by forming intermolecular disulfides with a substrate protein [76]. The redox state of PDI, and thus whether it is prone to catalyze oxidations or reductions, is controlled by other redox enzymes and by glutathione [75]. While most PDI is sorted to the endoplasmic reticulum (ER), significant amounts of PDI have also been detected associated with the plasma membrane, with the redox sites exposed on the cell surface [77], and secreted in an EV-associated form [52, 78, 79]. Importantly, PDI activity has been shown to be crucially involved in ATP-induced EV release by murine myeloid cells [52].

Several studies have implicated PDI family members as regulators of membrane fusion, making it conceivable that EV release and/or uptake can be mediated by PDI-dependent membrane rearrangements. For instance, sperm-egg fusion can be prevented by thiol scavengers and depends on expression of the PDI family member Erp57 on the sperm cell membrane and of the tetraspanins CD81 and CD9 on the egg cell membrane [80–82]. Notably, exosomal membranes are commonly associated with both tetraspanins and PDI, making it tempting to speculate that PDI may be involved in the fusion of tetraspanin-enriched membranes during EV biogenesis or uptake. Next to gamete fusion, the PDI family is also involved in the cell entry of several enveloped viruses, including retroviruses, whose biogenesis and cellular uptake bear striking resemblances to those of exosomes [83, 84]. PDI-catalyzed reduction of disulfide bonds in viral fusion proteins induces conformational changes which mediate fusion of the viral envelope with the host cell membrane [85, 86]. PDI may similarly regulate EV uptake by catalyzing thiol-disulfide exchange reactions in EV-borne fusion proteins. For instance, the fusion proteins syncytin-1 and syncytin-2 have been identified on EVs and proposed to mediate EV uptake by target cells [87]. The fusogenic
Table 3  Molecular targets with redox-sensitive thiol groups that regulate EV biology

| Molecular target                                                                 | Subcellular location | Mechanism of action                                                                 | Active form   | (Expected) Effect of oxidation/thiol blockage | Mechanism of EV release                                                                 | References |
|---------------------------------------------------------------------------------|----------------------|------------------------------------------------------------------------------------|---------------|---------------------------------------------|----------------------------------------------------------------------------------------|------------|
| Calcium channels (TRPA1, RyR, L-type channels, SERCA, IP3 receptors)             | Transmembrane        | Calcium influx into the cytoplasm inhibits flippases, activates SNARE-dependent membrane fusion and promotes calpain-and caspase-dependent cytoskeletal reorganization | Oxidized/adducted/nitrosylated | Increased EV release | Plasma membrane blebbing, fusion of MVE with plasma membrane | [60, 61, 67] |
| Calcium channels (SERCA, L-type channels, T-type channels)                       | Transmembrane        | Calcium influx into the cytoplasm inhibits flippases, activates SNARE-dependent membrane fusion and promotes calpain-and caspase-dependent cytoskeletal reorganization | Reduced      | Decreased EV release by activators of these channels | Fusion of MVE with plasma membrane | [60] |
| NSF                                                                              | Intracellular        | Recovers SNAREs for repeated rounds of membrane fusion                             | Reduced      | Decreased EV release | Fusion of MVE with plasma membrane | [69, 72–74] |
| PDI family members                                                              | ER, cell surface     | Recovers SNAREs for repeated rounds of membrane fusion                             | Reduced      | Decreased EV release | Fusion of MVE with plasma membrane | [52, 81, 83] |
| Thiol-rich fusion proteins (syncytin-1,-2, EFF-1, AFF-1)                         | Transmembrane        | Mediate membrane fusion                                                            | Reduced      | Decreased EV uptake | Involved in EV uptake (syncytin-1,-2), unknown (EFF-1, AFF-1) | [87, 109] |
| Phospholipid flippases                                                           | Transmembrane        | Maintain conical phospholipids (PS, PE) in inner membrane leafet                    | Reduced      | Increased EV release | Plasma membrane blebbing | [89] |
| Actin cytoskeleton                                                               | Intracellular        | Retraction of membrane blebs                                                       | Reduced      | Increased EV release | Plasma membrane blebbing | [67, 95] |
| Calpains                                                                        | Intracellular, can be membrane-associated | Degrade actin cytoskeleton and thereby prevent retraction of membrane blebs         | Reduced      | Decreased EV release | Plasma membrane blebbing | [97] |
| Cell surface thiols                                                              | Cell surface         | Unknown                                                                            | Reduced/oxidized (conflicting data) | Increased EV release? | Unknown, EVs can have microvesicle or exosome-like properties | [41, 47, 52] |

AFF-1 anchor cell fusion failure 1, EFF-1 epithelial fusion failure 1, EV extracellular vesicle, ER endoplasmic reticulum, IP3 inositol-1,3,5-triphosphate receptors, MVE multivesicular endosome, NSF N-ethylmaleimide-sensitive factor, PDI protein disulfide isomerase, PE phosphatidylethanolamine, PS phosphatidylserine, RyR ryanodine receptor, SERCA sarco/endoplasmic reticulum Ca^{2+}-ATPase, SNARE soluble NSF attachment protein receptor, TRPA1 transient receptor potential A1
activity of syncytins depends on several highly conserved cysteine residues [88], supporting the notion that they may be subject to thiol oxidoreductase regulation.

**Phospholipid flippases and scramblase**

Phospholipid flippases and scramblase are enzymes that regulate the proportions of various phospholipids in the inner
Retractive actin filaments and calpains

Under physiological conditions, membrane blebbing is counteracted by the retractive force of cytoskeletal actin filaments, whose activity depends on reduced thiols [93, 94] (Fig. 2e). Cytoskeletal inhibition by membrane-permeable thiol scavengers results in rapid and abundant shedding of plasma membrane-derived microvesicles (reviewed in [95]). The EVs induced by this treatment are very large (5–15 μm in diameter) and are thought to reflect the cytosolic and plasma membrane composition of their cells of origin [39, 95]. However, such large EVs rarely occur under physiological conditions. Thus, it remains to be elucidated whether blocking of cytoskeletal free thiols contributes to normal EV biogenesis. Thom et al. reported that exposure of neutrophils to CO₂ results in increased activity of inducible nitric oxide synthase (iNOS) and, consequently, in S-nitrosylation of actin. Abrogation of actin S-nitrosylation by UV-light prevented the EV induction, suggesting that the S-nitrosylation was required for CO₂-induced EV release. Intriguingly, the authors found that S-nitrosylated actin was associated with phospholipid flipase and PDI, which may further contribute to thiol-dependent regulation of EV release as discussed above.

Retraction of membrane blebs is additionally regulated by calpains, cysteine proteases that degrade actin filaments. Calcium-dependent activation of calpains prevents retraction of membrane blebs and promotes microvesicle formation [91, 96]. However, while calpains are activated by calcium, they become inactivated when their free thiols are oxidized. Therefore, calpain-dependent microvesicle release may be enhanced by membrane impermeable thiol-reactive compounds that activate calcium influx channels at the cell surface, but inactivated by thiol-reactive compounds that enter the cell and oxidize the active site thiols of calpains. Congruently, Dachary-Prigent et al. have shown that the membrane-permeable RCS N-ethylmaleimide and diamide inhibit calpain function and thereby prevent the release of platelet EVs in response to the calcium ionophore A23187 [97].

Cell surface-exposed thiols

A number of recent studies have proposed that the redox state of cell surface-exposed thiols is involved in the regulation of EV release [41, 47, 52]. Firstly, Furlan-Freguia et al. have shown that stimulation of murine macrophages with the danger-associated molecular pattern (DAMP) ATP results in P2X7 receptor-dependent upregulation of free thiols at the cell surface and in ROS-dependent release of thiol-rich microvesicles [52]. Cell pre-treatment with the membrane impermeable thiol-scavenger DTNB, as well as inhibition of PDI reductase activity (using the anti-PDI clone RL90)
prevented the microvesicle induction in response to ATP [52]. In contrast, inhibition of PDI oxidase activity (using the anti-PDI clone 34) directly induced increased cell sur-
fice thiols and thiol-rich microvesicle release [52]. A possi-
ble interpretation of these findings is that PDI may maintain
thiols in an oxidized state under control conditions, thereby
preventing microvesicle formation. Upon ATP exposure,
PDI activity may then shift from oxidase to reductase activity,
resulting in the appearance of free cell surface thiols and
subsequent ROS-dependent induction of thiol-rich microves-
icles. Similarly to Furlan-Freguia et al., Szabó-Taylor et al.
have found that stimulation of human monocytes with pro-
flammatory stimuli, namely lipopolysaccharide (LPS) or
tumor necrosis factor (TNF)-α, caused an upregulation
of cell-surface-exposed thiols [47]. However, the EVs that
these cells secreted were poor in exofacial thiols [47], in con-
tast to the thiol-rich EVs observed by Furlan-Freguia et al.
While Szabó-Taylor et al. did not assess whether there was
a quantitative change in EV release when monocytes were
stimulated with LPS or TNF-α, they did hypothesize that the
shedding of thiol-poor EVs may be a protective mechanism
to maintain the cell surface in a reduced state [47].

Data from our group further corroborates that the redox
state of exofacial thiols regulates EV release [41], although
part of our results appear to disagree with findings of the
two other groups. We found that treatment of airway epi-
thelial cells with the RCS acrolein, but not with the ROS
H2O2 causes depletion of cell surface thiols. Acrolein as well
as the membrane impermeable thiol scavengers DTNB and
bacitracin triggered increased release of small EVs express-
ing the exosome markers CD63 and CD81, whereas H2O2
had no measurable effect on EV release. The EV induction
appeared to be directly caused by the depletion of cell sur-
face thiols rather than being associated with the cell’s adap-
tive antioxidant response because EV induction required
continuous presence of the RCS, whereas a transient RCS-
stimulation was sufficient to induce upregulation of the cel-
lar antioxidant glutathione [41]. In our study, neither the
anti-PDI clone RL90 nor the PDI-inhibitor rutin affected
basal or RCS-induced EV release. Although we were unable
to identify a specific exofacial target protein of thiol modi-
fications, we could conclude that depletion of cell surface
thiols is sufficient to elicit an increased EV release in airway
epithelial cells.

Although all three studies provide evidence that cell
surface thiols may be crucially involved in the regulation
of EV release, a number of discrepancies remain. While
Furlan-Freguia et al. argue that an increase in the number
of cell-surface thiols is required for EV induction [52], we
found that depletion of exofacial thiols enhances EV release
[41]. Another discrepancy is that EVs released by cells with
increased cell-surface thiols were rich in exofacial thiols in
the study of Furlan-Freguia et al. [52], while they were poor
in exofacial thiols in the study of Szabó-Taylor et al. [47].
Additional research is required to reveal whether the differ-
ces between studies are due to the stimuli, the cell types,
the EV subpopulations or the exposure times that were
investigated. It has to be noted that oxidative processes were
involved in the cell surface thiol-dependent EV release in
all three studies. Thus, initial upregulation of reduced cell
surface thiols may be a prerequisite for subsequent oxidant-
dependent thiol modifications and EV induction. It should
be investigated whether the amount of cell surface thiols
regulates EV release via one or more specific thiol-bearing
proteins, or whether a more general mechanism is involved,
such as disulfide cross-linking of exofacial thiol-bearing pro-
teins by ROS or formation of bulky adducts by RCS, both of
which may influence membrane curvature.

**Thiol protection to prevent EV modifications: therapeutic implications**

As thiol modifications appear to modulate the formation
and functions of EVs upon cell exposure to pro-oxidant condi-
tions, thiol protection may be a promising strategy to prevent
detrimental changes in EV signaling under such conditions.
Indeed, several thiol-bearing small molecules, such as NAC;
NACA and glutathione are able to prevent EV induction
by a variety of ROS, RCS and pro-inflammatory stimuli,
likely by scavenging thiol-reactive compounds and prevent-
ing them from reacting with cellular thiols [41–43, 52, 53,
98–100]. NAC also inhibits EV-associated release of TF and
PS by cells exposed to oxidant conditions and consequently
decreases the procoagulant potential of EVs [42, 52, 53]. It
may also prevent EV-dependent secretion of pro-inflamma-
tory molecules [98], although this has been less well studied.
Importantly, NAC treatment appears to restore EV secretion,
composition and functions to the level observed for unex-
posed cells, rather than completely inhibiting EV signaling.
Thus, NAC may specifically prevent oxidant-induced deter-
imental changes in EV signaling without interfering with the
physiological functions of EVs.

In lung disease, particularly chronic obstructive pulmo-
nary disease (COPD), NAC is currently used as a mucolytic.
According to recent meta-analyses, NAC is associated
with improved small airway function and decreased exacer-
bation frequency in this target group when admin-
istered orally at ≥1200 mg/day [101–103]. Importantly, it
has been proposed that these clinical benefits can at least
partly be attributed to antioxidant and anti-inflammatory
properties of NAC or its thiol-bearing metabolites rather
than to the mucolytic activity alone [102, 104]. Additional
research could reveal whether prevention of cell-
ular thiol modifications and subsequent changes in EV
signaling contribute to this alternative mechanism of
action. Importantly, NAC has recently been proposed to be of clinical benefit in other conditions that are also hallmarked by inflammation and oxidative stress, such as insulin resistance and possibly neurological disorders [105, 106]. There is even early stage evidence suggesting an anti-thrombotic effect of NAC treatment [104, 107, 108]. This is in line with the observation that NAC prevents the release of procoagulant EVs in the response to oxidative thiol modifications [42, 52, 53]. Translational studies are required to elucidate whether inhibition of thiol-dependent EV modifications contributes to the clinical benefit of NAC in COPD patients and to determine whether additional target groups may benefit from NAC treatment due to this mechanism of action.

**Conclusion**

Taken together, protein thiols play a crucial role in the modulation of membrane fusion and membrane blebbing. Thereby, they regulate EV release and, possibly, uptake. EV release under thiol-depleting conditions may have evolved as a beneficial adaptive response to cellular oxidative stress. However, these EVs may also exert detrimental pro-inflammatory and prothrombotic effects. Additional research is required to establish the importance of thiols in EV biology and to identify the molecular mechanisms that mediate the thiol-dependent regulation of EV-related membrane rearrangements. Many known inhibitors of EV signaling interfere with vital cellular processes, making them unsuitable for the therapeutic modulation of EV signaling. Thiol-containing antioxidants such as NAC counteract the induction of EVs by pro-oxidant stimuli in vitro. Clinical studies are required to investigate whether inhibiting the release of pro-inflammatory and procoagulant EVs contributes to the therapeutic benefit of NAC in conditions of chronic inflammation and oxidative stress. Additionally, future research should focus on identifying the specific thiol-dependent mechanisms that are involved in the regulation of EV release and uptake, as these may be promising targets for specific pharmacological modulation of EV signaling.

**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

**Funding** This work was supported by the Netherlands Organization for Scientific Research (NWO) Grant 022.003.011 with the title “NUTRIM NWO Graduate Program: Metabolism and chronic disease” awarded to BJB.

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