



Effects of stress on endocrine and metabolic processes and redirection: cross talk between subcellular compartments

T.H. Elsasser*, S. Kahl, A.V. Capuco, W. Schmidt

United States Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705, USA

Received 13 February 2012; received in revised form 16 April 2012; accepted 16 April 2012

Abstract

Recent advances in genome analysis and biochemical pathway mapping have advanced our understanding of how biological systems have evolved over time. Protein and DNA marker comparisons suggest that several of these systems are both ancient in origin but highly conserved into today's evolved species. However, remnants of some of the more ancient functions of these chemical systems can run in conflict with the functions that those same pathways serve in complex organisms and tissue systems today. Relevant to the present topic, nitric oxide (NO) and superoxide anion ($O_2^{\bullet-}$), ancient cellular molecules in evolutionary terms, are recognized today as both necessary for the well-being and stable health of cells but also injurious to cells as elaborated in conjunction with the cellular stress response. Why the dichotomy? This question underlies one of the basic issues challenging researchers as well as practitioners in their approach to disease management. The fundamental proinflammatory response of the innate immune system of the host is needed for pathogen control but can be injurious to tissues from "collateral damage" from NO- and $O_2^{\bullet-}$ -derived reactive molecules capable of affecting protein function via post-translational chemical modification. This review highlights newer aspects of the biochemistry of the NO- and $O_2^{\bullet-}$ -mediated innate proinflammatory response and further show how protein and tissue damage via overproduction of reactive nitrogen and oxygen intermediary molecules such as peroxynitrite ($ONOO^-$) might be targeted to specific epitopes of proteins. Changes in the regulation of metabolism in response to proinflammatory disease states are discussed for GH signal transduction and tissue specificity.

Published by Elsevier Inc.

Keywords: Proinflammatory stress; Nitric oxide; Superoxide anion; Protein nitration; Mitochondria; Membrane

1. Introduction

Farm management and veterinary practices aimed at sound animal health can be thought of as a balancing act among production, protection, and intervention. It is well recognized that much of the tissue damage that arises in the host response to pathogen challenge is not so much the direct effect of the invading organism on

the tissue but rather the imbalance between pro- and anti-inflammatory components of the host cellular response to the detection of the immune stress [1–3]. When properly functioning, this "immune struggle" within the innate arm of the immune response generates a myriad of communicating molecules in a cascade of processes. It is initiated with pathogen recognition and progresses through a series of cytokine, arachidonic acid-derived, and free radical and reactive anion intermediates that further invoke the anti-inflammatory mediators to (hopefully) resolve in homeostatic balance. This comes at a price, however, when collateral damage to host tissue arises from the same mediators that were called on to kill invading organisms.

* Corresponding author at: USDA Agricultural Research Service, Bovine Functional Genomics Laboratory, Bldg 200, Rm 211, Beltsville Agricultural Research Center-East, Beltsville, MD 20705, USA. Tel.: +1 301 504-8281; fax: +301-504-8623.

E-mail address: theodore.elsasser@ars.usda.gov (T.H. Elsasser).

When we envision the concept of innate system response to infection stress, typically, we see a picture composed of responder cells (monocytes, macrophages, neutrophils, etc.) recruited to and infiltrating among function-specific organ cells (hepatocytes, secretory epithelial cells, muscle cells, mammary epithelium, etc). Responding cells function in terms of receptors that bind to foreign antigenic molecules particular to specific types of organisms (termed pathogen-associated molecular patterns, or PAMPs) and the more recently characterized endogenous stress signals termed “danger-associated molecular patterns” (DAMPs) [4–6]. The PAMPs are more recognizable in the process of inflammatory stress signaling as the toll-like receptors (TLRs) of which there are presently nine variations (TLR-1 to TLR-9). Bacterial disease in particular is detectable by responder cells via TLR-2 (recognizing peptidoglycan components of Gram-positive bacteria) and TLR-4 (recognizing lipopolysaccharide/endotoxin of Gram-negative organisms). The TLRs further associate with adapter molecules such as MyoD-88 to fine-tune the initiation sequence that starts the acute phase response. However, it is not the intent of this review to describe in detail the workings of these response systems. Rather, we report on the chemical nature of the responding cell-mediating products and show how attributes of the more ancient response mechanisms of stress detection and defense [altered membrane fluidity and the generation of nitric oxide (NO) and superoxide anion ($O_2^{\bullet-}$)] are the common underpinnings of the evolved response mediated by PAMPs, DAMPs, and other communicating molecules. In addition, we focus more intently on a specific aspect of immune stress-mediated perturbation in tissue function, namely, the effect of nitrooxidative stress—the set of biochemical events that occurs as a result of improper reactions between NO and $O_2^{\bullet-}$ that culminate in the post-translational chemical modification to proteins termed tyrosine nitration. Presently, there are more than 70 diseases ranging from chronic obstructive pulmonary disease to vascular atherosclerosis and progressive sepsis characterized as having aberrant protein nitration as a critical determinant of cellular malfunction [7–9].

2. Protein nitration as a basis for cell damage: NO-mediated post-translational chemical modification of protein tyrosine

Inflammatory stress is most often characterized by and referred to by the term “oxidative stress” in regard to perturbations in cell function and observed increases in apoptotic and necrotic events. More recently, how-

ever, significant attention has been directed toward a series of reactive events in cell stress that stem from the opportunistic interaction of two highly reactive stress intermediate molecules NO and $O_2^{\bullet-}$. When generated in aberrant proportions, these two molecules interact to make a third highly reactive oxynitrogen intermediate called peroxynitrite ($ONOO^-$) [7–9]. Termed “nitrosative stress,” the end result of the attachment of a nitrate group from $ONOO^-$ onto select amino acids such as tyrosine (forming 3'-nitrotyrosine, NT) is an alteration in protein function whose effects can range from complete inhibition to aberrant increased activity of the affected protein [10] along with concomitant changes in cellular health.

The main path toward protein tyrosine nitration is shown in Figure 1. Nitric oxide and $O_2^{\bullet-}$ generation increase in response to PAMP/DAMP/cytokine signaling (especially tumor necrosis factor- α , TNF- α) in function-specific organ cells as well as infiltrating responder cells (all have PAMP/DAMP/cytokine receptors) and proinflammatory mediators that initiate and

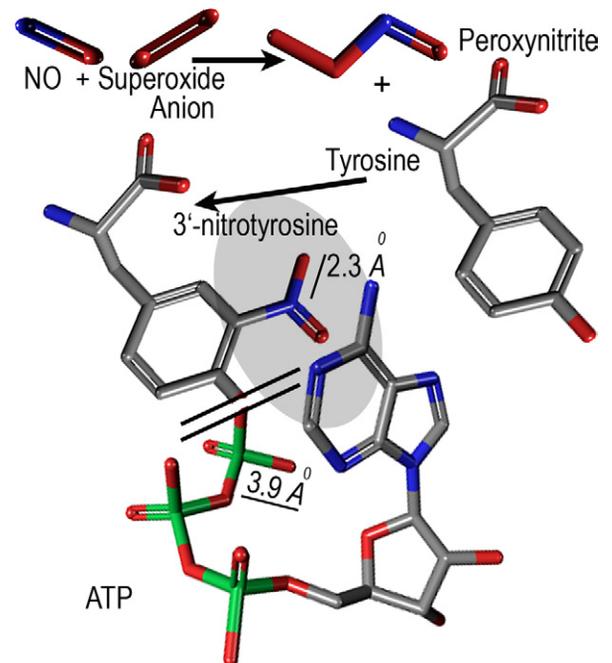


Fig. 1. Molecular model of the reaction between nitric oxide (NO) and superoxide anion ($O_2^{\bullet-}$) needed to generate peroxynitrite ($ONOO^-$) which, when formed, reacts spontaneously with favorable targets such as the 3' site of the phenolic ring of tyrosine (forming 3'-nitrotyrosine). Spatial and charge hindrances (suggested by the gray shaded region) significantly interfere with the ability of ATP to access tyrosines on kinases such as Janus kinase-2 critical for the slower (by “molecular time”) enzyme-driven transfer of the terminal phosphate needed for phosphorylation activation of this protein.

regulate the proinflammatory cascade through the acute phase response. In stark contrast with being generated to a lower degree in health-stable cells as a part of the normal intercompartmental signal transduction process, it is when these nitration compounds and by-product molecules are produced in high concentrations in confined subcellular compartments such as the mitochondria and membrane caveolar domains that cell damage occurs [1].

The confined space of the microdomain limits the diffusion potential of the reactants with a resulting increased probability of combining to form ONOO^- (reaction rate constant $\sim 10^{10} \text{ M}^{-1} \times \text{s}^{-1}$ [7]) outcompeting diffusion-limited interactions with other less reactive targets. Once formed, ONOO^- can react either directly with target tyrosine residues or can form an even more reactive nitrating species in the presence of increased CO_2 levels, nitrosoperoxy carbonate [11]. The pathway to this reactant is significant in systemic proinflammatory illness in that under the influence of cytokine-, prostaglandin-, and NO-mediated blood flow shunting, localized areas of anoxia and ischemia can develop the increase in the local partial pressure of carbon dioxide (PCO_2), favoring the accelerated formation of protein nitration via nitrosoperoxy carbonate [12]. Significant to the process of tyrosine nitration, the charge differential added to tyrosine as a consequence of the nitrate moiety added at the 3' position has substantial effect on the immediate local epitope structure of a peptide or protein. Critical changes associated with nitration include rearrangement of hydrogen bonds and van der Waal and charge interactions that manifest themselves as special boundary impediments to free rotation and further obligatory chemical modifications. In reference to Figure 1, it is readily apparent that the addition of the nitrate to the phenolic ring of the tyrosine (spatial orientation of a flat triangle 2.3 Å on a side) can result in the complete blockage of further attachment of phosphate at the ortho-position (spatial orientation of a pyramid with sides 3.9 Å), a kinase-driven event necessary for the transfer of the terminal phosphate from ATP to the tyrosine activation site of many proteins, signal transduction, and mitochondrial proteins in particular [7,13,14]. Because of the preferential Gibb's free energy of reaction, the direct chemical nitration of tyrosine via any of the ONOO^- reactants outcompete enzyme-driven (kinase) phosphorylation modifications. The actual process of tyrosine nitration is not a random event. Several proteomic analyses indicate that not all tyrosine residues in a molecule are capable of being nitrated. The basis for the targeted tyrosine nitration

has been addressed in research by Souza et al [15] and Lanone et al [16].

3. Sensing cellular stress: origin of the cellular stress response

3.1. Orientation overview

From the standpoint of a biochemically significant effect, what we call a "breakpoint" that is simply the critical level of structural change in a physical constituent of a cell (a protein, a lipid, an organelle, etc.) at which a level of perturbation is reached that constitutes harm to the function of the molecule or subcellular structure [1,12]. From a molecular standpoint, three overarching principles converge at the intracellular (subcellular) structure–function relation to confer on the cell the capacity to sense harmful conditions in a process termed "breakpoint stress recognition." These principles are grounded in the physical laws that govern lipid fluidity, protein stability, and chemical reactivity as a function of concentration, distance, half-life, and interaction. One part of this stress recognition is carried over from the cell's ancient past and can be termed "membrane-centric." A membrane-centric response can be thought of as a change in the membrane's three-dimensional stability as might arise from intercompartmental differences in pressure and/or the molecular torque stresses imposed on lipids and proteins in the membrane. At the heart of this is the basic character of lipid fluidity. The membrane can be the plasma membrane or compartmental membranes demarcating mitochondria, endoplasmic reticulum, Golgi bodies, etc. These changes are observed as alterations in how freely the hydrophobic tails of membrane fatty acids interact with other membrane components such as proteins, cholesterol, and phosphatidyl constituents. Changes in membrane fluidity need occur in specific membrane domains and can arise as a result of pH, heat, cold, ionic strength and red-ox character [17]. In another form of stress recognition, cells developed the capability to detect when de novo synthesized proteins are formed incorrectly and toward this detection capacity, intracellular stress causes protein misfolding and aggregation. This latter self-assessment is referred to as the "unfolded protein response," UPR [18–20]. The final component of the triad is chemical in nature and reflects how reactive nitrogen and oxygen intermediates interact when generated in the respective subcellular compartments. The fundamentals of a cell stress recognition model, summarized in Figure 2, are elaborated on in the following subsections.

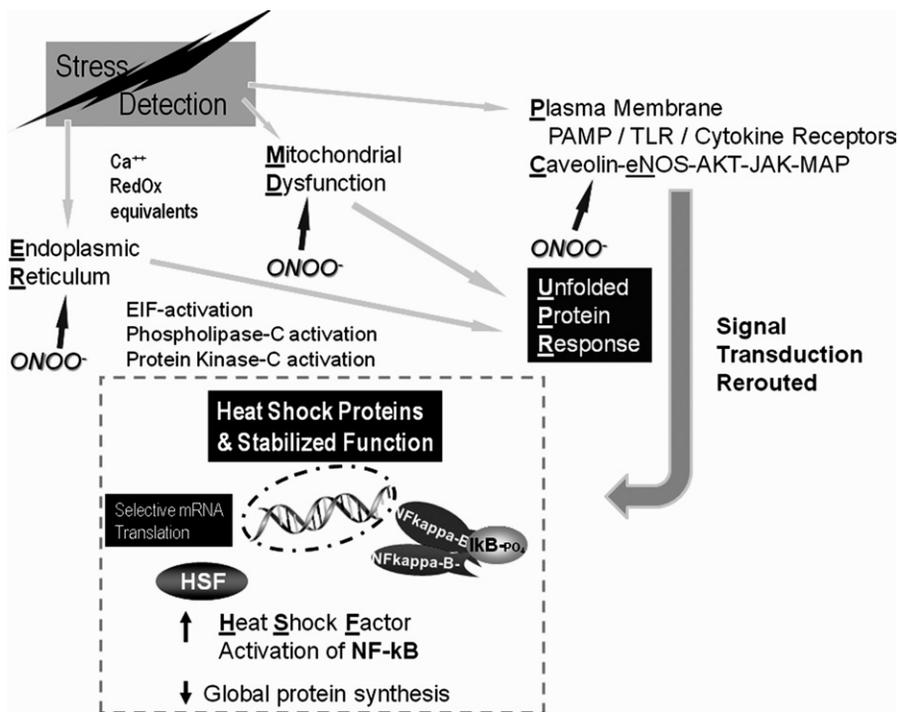


Fig. 2. The highly structured order of cellular microdomains facilitates the positioning of stress response intermediary molecules to permit the untimely generation of locally high concentrations of peroxynitrite (ONOO^-). Because of the high reactivity of this oxynitrogen anion with both membrane lipids and subcellular organelle proteins, chemical modifications to the membrane fatty acids and organelle proteins lead to changes in membrane fluidity as well as protein aggregation and fuel the cell stress response in terms of the UPR (unfolded protein response) in several subcellular membrane-bound compartments. Direct post-translational chemical modifications to signal transduction proteins also redirects hormone and cytokine signaling that can affect processes that range from the capacity to recover through apoptosis and necrosis.

3.2. Lipid bilayer compartments and membrane fluidity

Before we can appreciate the extent of the effect inflammatory stress-related protein nitration has on cell function, it is important to understand the origins of the cell stress response and then visualize how the chemical modification (nitration) of cytoplasmic, membrane, and intracompartamental proteins affects this response. The first characteristic identified to define a potential for life was the compartmental differentiation of an “inside” vs the rest of the world “outside” as mediated by a membrane. It is not surprising therefore that the basic sensing of distress by some version of a cell would be manifested first in a change in some aspect of the integrity of the boundary demarcating inside vs outside, followed by the generation of a response to mitigate the effect of the stress on the boundary integrity. The boundary is typified by the fluid mosaic lipid–protein bilayer as originally modeled by Singer and Nicholson in 1972 [21]. The evolved animal cell is fundamentally a bundle of membrane-bound structures we call the organelles, and, collectively, these carry out the basic needs of

the cell for the greater purpose of supporting the life and regeneration of the organism. In the bilayer, hydrophobic portions of the fatty acids sit in proximity to the aqueous external and cellular internal environment, whereas the hydrophobic tails of the fatty acids orient toward each other, generating a polarity of structure that can be characterized in terms of how flexible (referred to as fluidity) the hydrophobic tails are under different conditions of heat, cold, and chemical modification [22–24]. Altered lipid fluidity; that is, the ease in which membrane-based molecules move in-plane (*cis*-movement, lateral motion in the same plane of the bilayer) or across plane (*trans*-movement, flip-flopping from one layer of the bilayer to the other), dictates the basic rate at which intramembrane constituent (lipids, phospholipids, proteins, etc.) can interact or change conformation themselves.

The next higher level of organization, membrane asymmetry (where the membrane matrix differs in lipid composition between cell types and subcellular organelle compartments) was well characterized by Lenoir et al [22] in regard to how membranes have different functions and are capable of responding to different

forms of stress. Biochemically speaking, these stresses, whether physical, chemical, or immunological, manifest their effect by altering the thermodynamic stability of discrete locations in the membrane. These locations may be particular to specific organelles or microdomains of membranes characterized as lipid rafts, caveolae, and membrane regions with enhanced cholesterol trafficking. For a more detailed review of this topic the reader is referred to the recent article by Fessler and Parks [25].

3.3. Unfolded protein response

Cellular metabolism ultimately depends on the proper functioning of proteins, and several systems have evolved to ensure that the conformation and integrity of proteins are monitored and maintained throughout the cytoplasm, mitochondria, and the endoplasmic reticulo-Golgi system. In reference to Figure 2, each of these subcellular compartments has its own mechanism for identifying malformed/malfunctioning proteins, and each has its unique set of nuclear signaling factors that regulate gene expression appropriate to the sensed perturbation. The best understood and described theory of how cells sense that their equilibrium is perturbed (ie, stressed) is the ubiquitous UPR [18–20]. The UPR was first detailed for endoplasmic reticulum (ER) functioning (UPR^{endo}), but more recently the mechanisms governing the UPR have also been mapped for the mitochondria (UPR^{mito}) [26]. The single most important feature of the UPR^{endo} is that on initiation of this signaling response global protein synthesis in the affected and responding cell will be shut down with the exception of cells capable of generating survival proteins (chaperone proteins). The most important classes of survival proteins are the heat shock proteins followed by antiapoptotic proteins such as Bcl-2 [27]. The microenvironment of the ER is quite oxidizing in comparison with other regions and organelles in the cell, and it is also sensitive to challenges to the stability of its red-ox character [1]. Cells manufacture proteins in the ER as prescribed in the translation of mRNA and refine the tertiary structure of the protein in terms of folding with post-translational modifications, including the addition of sugars leading to the exportation to the Golgi network. High et al [28] and Ellgaard and Helenius [29] determined steps critical to the exportation of only properly folded proteins wherein two facilitator proteins (calnexin and calreticulin) recognize specifically α -(1,3)-glucose-mannose bonds at specific residues of asparagine to orient the peptide regions needed to form disulfide bonds. Then, because of the oxidizing nature of the compartment, the red-ox-sensitive disulfide isomerase ERp57 finalizes the orientation, and the

protein is exported. We recently reviewed the signaling components associated with the UPR that indicate that an inappropriate or harmful condition is present as recognized by the build-up of misfolded proteins and artifacts leading to proteotoxic stress [1].

Participating in this process, membrane-resident components of the ER membranes such as BiP (resembling IgH-chain binding protein) which recognized several ER stress response-activating proteins with the cascade progressing through protein double-stranded RNA activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) to which is attached the conformation-sensitive regulator of mRNA translation, eukaryotic initiation factor-2 α . Again, the signaling event is a phosphorylation of a critical control element, this time when PERK phosphorylates specifically the serine 51 residue on eukaryotic initiation factor-2 α . This action specifically stops globally in the cell mRNA translation by blocking the ability of S-methionine tRNA to bind to the ribosome. Furthermore, PERK also regulates the activity of activating transcription factor-6. Under PERK guidance, activating transcription factor-6 is cleaved from a 90-kD to a 50-kDa protein in further Golgi processing to release to the nucleus a set of gene response modifiers called ER response elements that specifically bind to regulatory response elements in the promoter regions of several genes associated with cell survival, including other disulfide isomerases, C/EBP (C-C-A-A-T/enhancer-binding protein) homologous protein (CHOP), GRPxx's and XBBp1, and the all-important heat shock proteins. The third component of the ER-based UPR is the resident kinase and endonuclease inositol-requiring enzyme-1 (IRE-1). The principle mechanism proposed in the attempt to understand how misfolded proteins activate the total UPR cascade is the BiP repression pathway. When bound to BiP, IRE-1 is inactive. The accumulation of misfolded proteins results in the displacement of IRE-1 and activation of IRE-1. In line with these events and serving as a redundant backup stress response system, sensed perturbations further alter lipid membrane fluidity, increasing the outward flux of Ca²⁺ from the ER, alters the stability of the ER red-ox environment, and increases phospholipase C and phosphokinase C activity. The sum of these events, therefore, drives the reorganization of protein synthesis priorities and the balance between antiapoptotic and proapoptotic events in the cell.

Proinflammatory stress (eg, that triggered by TLR/TNF- α -initiated apoptosis) as well as physical stresses (eg, consider fluid shear stress affecting vascular endothelial cells) is recognized as a major factor shaping the entire response of the cell via the intracellular signals (signal transduction elements, DNA transcription mod-

ifiers, histone protein modification, and free radical and reactive anion and cation intermediates) that further shape the detection of stress by the mitochondrial organelle itself. Fundamentally, four processes can be summarized to account for the mitochondrial response to stress: (1) increased permeability of the mitochondrial membranes that generate increased outward calcium flux and perturbed hydrogen ion pumping (with marked mitochondrial swelling), (2) increased generation of NO and $O_2^{\bullet-}$ in association with altered internal red-ox state and inefficient electron and charge transfer down the transport chain, (3) release of NO- and $O_2^{\bullet-}$ -chemically modified proteins such as cytochrome-C oxidase that serve as signaling molecules to the cytoplasm from the mitochondria, and (4) activation of a version of the UPR particular to the mitochondria, the UPR^{mito}. Two major factors regulate the intensity of the UPR^{mito}. The first is the proportion of proteins present in the mitochondria that originate from nuclear DNA that encode relative to the amount of protein present as generated from mitochondrial DNA encoding. The second is the degree to which oxidized and nitrated proteins aggregate [30]. In that stresses compromise the integrity of the electron transport chain, any disturbance in this electron flow results in increased $O_2^{\bullet-}$ and NO generation. Because of the close proximity of these reactants, protein-oxidizing and NO-based nitrosating reactants in particular (such as ONOO⁻ [7–10,12]) are generated, and the action of these on the nearby mitochondrial proteins leads to damage and aggregation. In comparison with the ER, mitochondria are uniquely challenged to send and receive stress signals from other locations in the cell because of their complex double compartment, multimembrane structure. In addition, because of the multimeric composition of many of the mitochondria electron transport chain components, there is a fine line between the detection of aggregated protein malfunction vs properly integrated multimer association of electron transport chain proteins such as Complex V–ATP-synthase [7,31]. In light of this complexity, it must be remembered that even in normal function situations, the need to control overproduction of $O_2^{\bullet-}$ and NO from both mitochondrial and cytoplasmic sources is a fine-tuned battle between normal signal transduction and pathology.

3.4. Nitric oxide- and superoxide anion-derived reactants chemically modify proteins and lipids in the cytoplasm and mitochondria

In comparing the old with the new, the ancient with the evolved, few messenger molecules have changed roles more than NO and $O_2^{\bullet-}$. The fundamental char-

acteristic that served to coordinate interactions between NO and $O_2^{\bullet-}$ (and in a sense prevent untimely interaction) was the short half-life of reactivity of each compound. With regard to molecular interactions within cells, the propensity for two molecules to react is first proscribed by spatial and temporal limits to interaction, and further definable in terms of how spontaneous the reaction is apt to occur (a “ $-\Delta G$,” Gibbs Free Energy < 0). Intracellular compartmentalization exists in structures such as caveolae and mitochondria and functionally favors spatial and temporal (and therefore concentration-dependent) molecular interactions. When these reactants are overgenerated as they are likely to be in times of proinflammatory stress, the reactants that are evolutionarily conserved are the ones most likely to cause intracellular harm. Nitric oxide is generated from arginine by any of four isoforms of NO synthase (NOS), endothelial-constitutively active NOS, neuronal NOS, inducible NOS, and mitochondrial NOS. In terms of the stress response all forms of NOS contribute to overt production of NO with some isoforms such as inducible NOS being considerable more active in infiltrating immune cells such as neutrophils, macrophages, and monocytes [7,9,12]. Superoxide anion, by contrast, is generated mostly in the mitochondria when during mitochondrial stress the disrupted flow of electrons down the electron transport chain fails to reach the terminal end point and the oxygen escapes as its free radical form. In addition, the enzyme xanthine oxidase (XO) is significantly up-regulated in tissues in response to inflammatory stress [32], and this enzyme is capable of generating cytoplasmic concentrations of $O_2^{\bullet-}$ of significant and relevant tissue concentrations [33]. To a significant extent the contribution of the XO effect on $O_2^{\bullet-}$ generation is tissue dependent. Although most cells have some XO activity, the liver hepatocytes [34] and bovine mammary epithelial cells [35] have extremely high XO activity levels. Although XO may have appropriate bactericidal properties [36], the resulting large concentration of localized and compartmentalized $O_2^{\bullet-}$ generated by it are thought to contribute to the capacity to make reactive-nitrating agents in vivo such as ONOO⁻. A factor complicating the simplicity of this observation is the fact that under some proinflammatory conditions the stable end-product of NO catabolism, that is nitrate, can actually be reduced by xanthine oxidoreductase back to active NO whereby the XO-derived NO can fuel the further generation of ONOO⁻, and the switch between the oxidizing and reducing capacity of the XO is determined by subcel-

lular microenvironments especially where affected tissues exist in a partial anoxic state [37].

In terms of generating a mitigating response to the perturbation stress, cells evolved the capacity to generate and release reactive oxygen and nitrogen molecules to both rapidly modify the internal and boundary membrane microenvironments of the cell and serve as a signaling molecule to juxtaposed cells. To this point, it has been experimentally observed that NO directly alters the fluidity of erythrocyte membranes through a pro-oxidant action [38], as well as changing the function of membrane-embedded enzymes via localized alterations in lipid fluidity [39]. Therefore, from an evolutionary standpoint, the fundamental basis for stress detection and response by a cell can be traced back to the most ancient criteria for survival. Researchers have determined that not all areas of the bilayer respond similarly to a stressor, and this is underscored with the present state of knowledge about the presence of stress detection foci being located in lipid rafts and caveolae [1,40]. Stresses imposed on membranes alter thermodynamic entropy of sensitive microdomain regions such as lipid rafts and caveolae (ie, the bilayer is too fluid or too rigid) which in turn reorder van der Waal's forces and hydrogen bonding [22–24]. In turn, these “neighborhood” domain changes increase or decrease tertiary structures of kinases in the membrane foci with actual physical compression or relaxation forces.

4. Strategies developed to identify and quantify critical nitration targets

There are a tremendously large number of potential tyrosine targets in a cell which could be affected by the generated mediators of nitration. To gain more specificity to identify potential nitration targets with relevant biological effect, we needed to have a strategy on which our investigation could focus with specific regard to critical control points in metabolism and growth. First, we needed to establish that for levels of proinflammatory stress encountered under “normal farm and husbandry conditions” tyrosine-nitrated proteins were appropriate as biomarkers of low-level, recoverable stresses. We implemented a well-established endotoxin challenge protocol 41 in addition to several parasite models to suggest what patterns of protein nitration occurred in critical metabolic-integrating organs such as the liver. Figure 3 was generated through immunohistochemical localization of nitrated proteins with the use of an affinity-purified antibody to NT. The figure shows the tyrosine nitration patterns of various

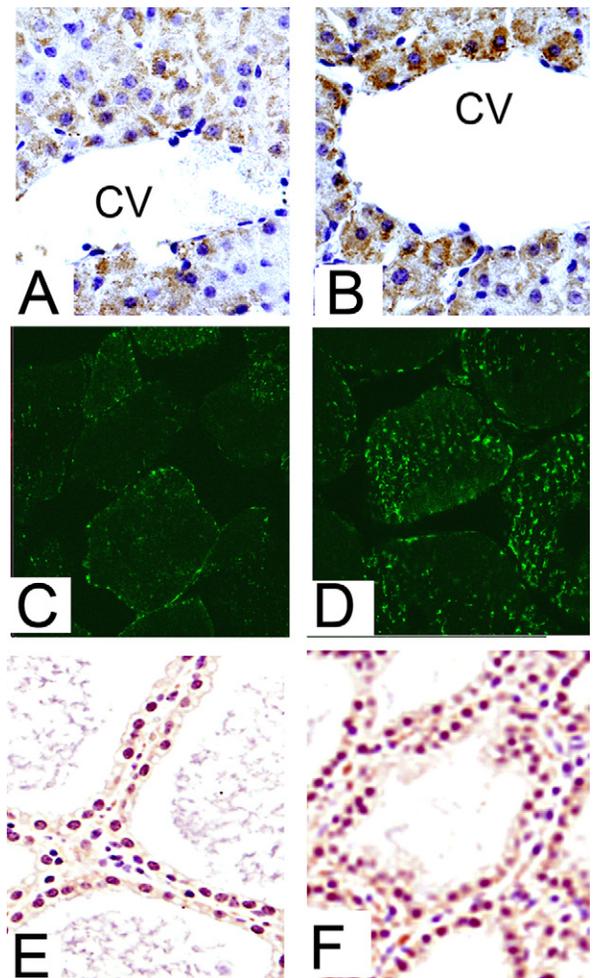


Fig. 3. The general presence of nitrated proteins can be determined with immunohistochemical techniques that use antibodies directed against 3'-nitrotyrosine (NT). Panels A and B (immunohistochemistry) indicate that hepatic generation of NT develops rapidly after lipopolysaccharide (LPS) challenge in calves in structural areas such as the central vein (CV), known to have high levels of mitochondria and therefore the capability of generating highly localized concentrations of nitric oxide (NO) and superoxide anion ($O_2^{\bullet-}$). In an experimental infection of calves with the muscle-resident parasite *Sarcocystis cruzi* (panels C and D, immunofluorescence) significant protein nitration occurs in muscle fibers commensurate with relatively high levels of atrophy and muscle wasting. The bottom panels (E and F) show the up-regulation of NT in mammary tissue of a cow infected with *Escherichia coli*. The image represents tissue collected 12 h after intramammary challenge with the *E. coli*. Note that at this time the response is principally that of the mammary epithelial cells in that the tissue is largely devoid of infiltrating somatic cells, and evidence of tissue damage is already apparent.

bovine tissues observed in liver from calves in response to an endotoxin challenge applied 24 h earlier (Fig. 3, A and B [41]), in psoas muscle from control calves and calves infected with *Sarcocystis cruzi* (a protozoan par-

asite) (Fig. 3, C and D [42]), and in mammary tissue of a control cow and cows infected with *Escherichia coli* (Fig. 3, E and F; unpublished observations - T. Elsasser, 2010). For the liver, the particular image is of a region surrounding a central vein (CV). The brown immunostaining (dimethylbenzidine plus horseradish peroxidase) is slight and diffuse in a control calf, whereas the intensity of the staining increases >10-fold after the proinflammatory influence of the bacterial toxin. Note the pattern of nitration in the lipopolysaccharide (LPS)-responding tissue (Fig. 3B) with a targeted higher increase in cells lining the CV that mitigates away from the CV in a gradient pattern. This pattern is consistent with the greater metabolic activity of the centralized cells and largely parallels the higher metabolic character and mitochondrial red-ox state in these cells [43,44]. Hence, the greater potential for proinflammatory levels of NO, O₂^{•-}, and nitrating reactants such as ONOO⁻. The immunofluorescent pattern of NT immunostaining in muscle fibers is shown (Fig. 3, C and D). Note the 7-fold increase in the presence of NT in tissue from the parasite-infected calf (Fig. 3D) and in particular the generalized spread of punctuate loci throughout the fiber also consistent with the observed patterns of mitochondria in slow-twitch, type 1 fibers [43]. Horseradish peroxidase immunostaining with Nova Red as substrate indicates that the mammary epithelial cells are targets for nitration in the early (12 h after infection) cellular response to coliform mastitis (Fig. 3, E and F). Particularly interesting is the increased diameter of cells displaying high levels of nitration indicative of edema, membrane barrier dysfunction, and generalized inflammatory condition. Collectively, the data indicate the utility of detection of generalized NT presence in proteins as useful as a biomarker of both modeled LPS and active infection stress.

However, in seeking more specificity in the nitration effect, we thought that a more-focused approach might be relevant to discriminating between what cellular systems were being affected by a given level of proinflammatory stress and, therefore, perhaps more relevant to a decision strategy toward suggesting an appropriate intervention. We chose a relevant protein for the initial focus because of its central role in growth, namely, transducing the GH signal between GH binding to its receptor and the nuclear appearance of the signal transducer and activator of transcription 5b (STAT-5b) dimer [13]. Janus kinase-2 (JAK-2) is a determinate of not only GH signal transduction in JAK-STAT processing but also because JAK-2 is a signaling element for a number of hormones and cytokines and TNF- α as well

[45]. In addition, it is known that these receptors are located in relevant plasma membrane caveolar cage structures along with the needed source of NO, endothelial-constitutively active NOS [46]. According to the data of Feng et al [47], the most significant phosphorylation activation of JAK-2 toward the capacity for GH to initiate IGF-1 transcription and peptide release was the specific phosphorylation of JAK-2 at one or both the tyrosines located in the 1,007 and 1,008 positions of the primary amino acid sequence, and we targeted these for further nitration analysis.

It was important to us that, if we were to go down this investigational path and expend resources for reagent development to measure site-specific nitration in a protein, some indication be present that a nitration could influence in some physical manner the basic structure of the protein in a manner relevant to some aspect of perturbed biochemical function (critical control point?) of that protein. To accomplish this we used the process termed global energy-minimized three-dimensional structuring whereby atomic models of the peptides were constructed with computational chemistry (MOPAC or molecular orbital package) software (Alchemy 2000, version 2.0 Tripos Inc., St. Louis, MO, USA) with visual rendering of the structures accomplished with Hyper-Chem molecular imaging software (version 5.11; Hypercube Inc, Gainesville, FL, USA). When this information is coupled with data derived from high-field nuclear magnetic resonance analysis of these peptides in solution, information can be amassed on the angular and rotational change in the peptide region with particular regard to hydrogen bonding effects and alterations in α -helix structure. For JAK-2 we derived the amino acid sequences of peptides flanking the native, the nitrated 1,007 and 1,008 tyrosines, and phosphorylated 1,007 and 1,008 tyrosines, and subjected the peptide to the Hyper-Chem processing. The effect of the nitration and the planar-spatial requirements for the respective phosphorylation are shown in Figure 4 (from Elsasser et al [14]). In essence what was observed was that, with the addition of the nitrate moiety(s) to tyrosines at this phosphorylation epitope, a significant rotation of the tyrosines develops that challenges the planar-spatial shifts in the tyrosines needed for orientation when phosphorylated (Fig. 4, A–D). The nitrated molecule is observed as elongated and narrowed by several angstroms in comparison with its native or phosphorylated versions. Further, we observed that, based on Gasteiger-Marsili electrostatic modeling of the peptides, the aspartate and glutamate residues in positions 1,004 and 1,006 needed for hydrogen bond stabilization

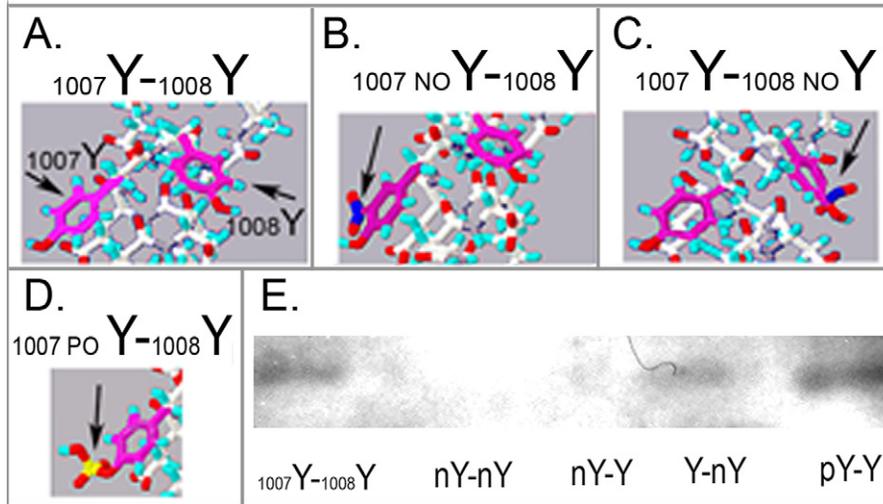


Fig. 4. Hyper-Chem renderings (A–D) visualize the effects of nitration on the rotation and accessibility of the 1,007 and 1,008 position tyrosines in Janus kinase-2 that compromise phosphorylation. The 3'-OH of the phenolic tyrosine structures are charge stabilized to aspartate and glutamate residues within the α -helical proline-to-proline loop. Phosphorylation of either or both tyrosines at their respective ortho-positions requires the symmetry of the helix, as stabilized by the increased affinity of the phospho-modified (requiring increased positive charge) tyrosines for the carboxyl groups of aspartate and glutamate as per the favorable change in the free energy at the site. Nitration at the 3'-tyrosine phenolic position results in a net negative charge that cancels the net positive charge of the phenolic, preventing affinity between the OH^- of either tyrosine and any COO^- , making the ortho position unfavorably presented for, as well as spatially compromised for, phosphorylation, functionally further making the molecule inactive for kinase activity. (Adapted from Elsasser et al [14].)

were disrupted, further negating kinase activity via perturbations in critical nearby glutamate and arginine residues. We further tested the biochemical effect of this chemical nitration modification by using native and nitrated variants of this epitope peptide as substrates in an *in vitro* tyrosine kinase phosphorylation assay. The results of this process (Fig. 4E) confirmed that the nitration of either the 1,007 tyrosine or both the 1,007 and 1,008 tyrosines was sufficient to totally block the capacity to phosphorylate these tyrosines. Nitration of the 1,008 residue alone was sufficient to decrease the phosphorylation of the 1,007 tyrosine by >50%.

With this information we developed a highly specific, affinity-purified antibody to the nitrated 1,007Y to 1,008Y JAK-2 epitope and used this for several applications, including Western blot analysis, immunohistochemistry, and confocal *in situ* hybridization [13,14]. Using this antibody, we developed data consistent with our phosphorylation evidence, indicating that cells in the liver that had developed nitration of JAK-2 in the 1,007Y or 1008Y position in response to a moderate proinflammatory challenge with endotoxin were incapable of generating a metabolic response to GH as assessed by *in situ* hybridization localization of mRNA for up-regulated IGF-1 gene expression. This effect was further determined to be associated specifically with caveolar-bound constitutively active NOS (by

both Western blot analysis of anticaveolin-2-immunoprecipitated proteins and double antibody confocal microscopy) and down-regulated when arginine antagonists were present to decrease the cogeneration of NO contiguous with a direct increase in sustained STAT-5b phosphorylation, dimerization, and translocation to the nucleus, indicative of progressive GH signaling to the nucleus via JAK-2. The *in vivo* generation or nitrated JAK-2 is shown in Figure 5. Calves challenged with endotoxin generate high levels of hepatic-nitrated JAK-2 contemporary with the progressive loss of responsiveness to GH [47,48]. The enlarged view of the image analysis-processed immunohistochemical images (red pixels indicate the specific nitrated JAK-2 antigen in the affected tissue) in the bottom panel of Figure 5 clearly indicate that nitration inactivation at this epitope location occurs in this signal transduction protein critical to the GH pathway. This impact point was further shown to contribute to mechanisms associated with the arrest of GH anabolic signaling as occurs during endotoxin-mediated proinflammatory stress. The data generated from pixel density image analysis indicated that, as a group, LPS-challenged calves mounted a response 15-fold higher than that present in nonchallenged calves. At the present time we are working out the details needed to isolate and functionally characterize purified nitrated and native variants of

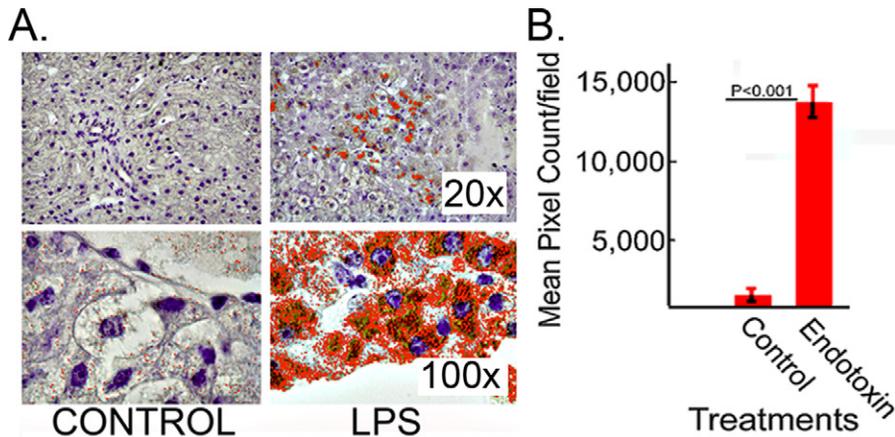


Fig. 5. Antibody-generated specifically to the nitrated 1,007 and 1,008 tyrosines of Janus kinase-2 (JAK-2) was able to define and characterize the generation of nitrated JAK-2 in calf liver tissue in response to a proinflammatory endotoxin challenge. Images on the far left were derived from a control, saline-challenged calf, and those on the right were from the endotoxin calf. The top panels of the micrographs represent low-power ($\times 20$) observation, and the lower panel shows high-power ($\times 100$) observation. The red dots were generated by the Image-Pro quantitative image analysis program whereby the specific nitrated JAK-2 pixels were accounted for. When averaged across six calves per treatment, the presence of the proinflammatory response to endotoxin generated a 15-fold increase in cellular nitration.

other proteins critical to energy and hormone/cytokine signal processing to determine their effects on the shift from anabolic to catabolic status during illness. Suffice it to say that the implementation of computational chemistry coupled with nitration epitope recognition and epitope function domain analysis is a powerful tool capable of identifying putative nitration targets with effect on pathophysiology.

To bring this review full circle, the opening concept of cell microdomains needs to be addressed with reference to our developed theme of protein nitration changes that accompany the proinflammatory state in food producing domestic animals. Figure 6 summarizes the type of information we have gathered on where in the cell certain aspects of protein-specific tyrosine nitrations tend to occur. Information of this sort can then be interpreted toward developing intervention strategies to limit the extent of proinflammatory tissue damage while preserving the capacity for the natural immune system to challenge pathogens.

An interesting feature of our approach is that we can consider a proinflammatory response a proinflammatory response without a real need to classify the origin in regard to what the pathogenic cause is. Whether parasitic, bacterial, or toxin-derived stress is present, the generation of aberrant nitrated proteins is key to understanding how the tissue damage is occurring. In regard to nitration of mitochondrial proteins in end-stage parasitic muscle disease, confocal colocalization of antigens associated with nitration (using anti-NT antibody) and Complex V- α subunit of the mitochon-

drial ATP-synthase (using anti-Complex V- α subunit antibody) indicated that the most nitrated proteins in muscle can be tracked back to the mitochondria themselves as the structure-associated endogenous sources of both NO and $O_2^{\bullet-}$ (Fig. 6, A–C). In this instance, it is interesting to note that the main effect of the disease stress is significantly more evident in slow-twitch, type I muscle fibers, the fibers with the highest density of mitochondria [42].

Referring back to the earlier example of the nitration of JAK-2, we were also able to localize this event to the subcellular plasma membrane microdomain of the caveolae [13]. The localization of this specific nitration to the caveolae (Fig. 6, D and E) underscores the importance of membrane-centric responses where the resident content of constitutively active NOS (a caveolin-caged protein) along with cytoplasmic XO-derived $O_2^{\bullet-}$ or that which had escaped from perturbed mitochondria serve to cogenerate the nitrating reactant(s).

For the mammary gland, the capacity to generate one of the critical limiting reactants leading to $ONOO^-$ production may be inherent to this gland and perhaps predispose it to these nitrooxidative stresses. The mammary gland is rich in XO [46]. The data in Figure 6, F and G, summarize recent findings on a clinical case of naturally acquired *Klebsiella* (tissue specimens from USDA Veterinary Animal Pathology Report). The photomicrographs indicate that the cells presenting the higher levels of protein nitration (Fig. 6F) can be localized to cells also presenting the greater increases in epithelial XO (Fig. 6G), consistent with the need for a

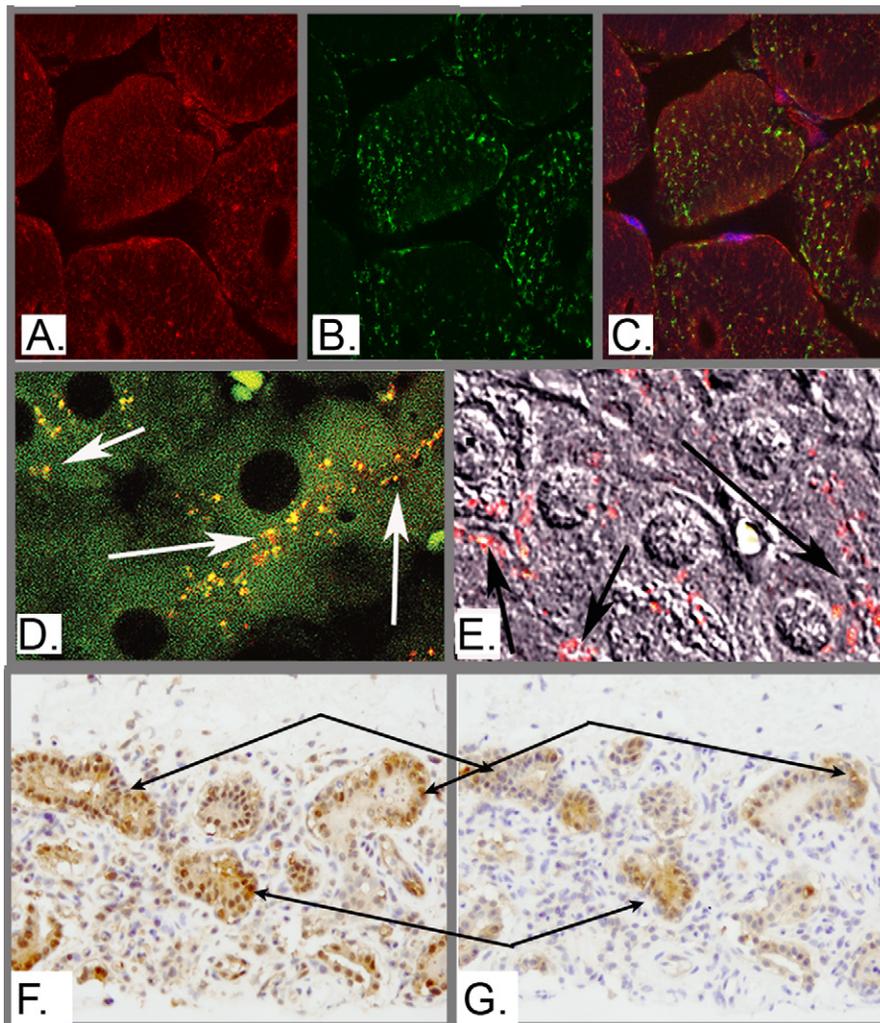


Fig. 6. Immunohistochemical and immunofluorescent colocalization of nitration targets to subcellular organelle matrix components. Panels A through C show in muscle fibers (from *Sarcocystis cruzi*-infected calves) that the up-regulation of nitrotyrosine (nitrotyrosine antibody; panel A) colocalized in the mitochondria (complex V antibody; panel B) as seen in the overlay image (panel C, yellow-green pixels). The nitration of Janus kinase-2 (JAK-2) (panel D, anti-nitrated JAK-2 serum) in liver tissue from endotoxin-challenged calves was colocalized (dual-color confocal imaging) to caveolae (using anticaveolin-1 antibody) and even more clearly to membrane subdomains when photographed with an interference phase-contrast image (panel E). The use of immunostained serial sections of mammary gland facilitated the identification of cells that generate cytoplasmic signals for both nitrated proteins (panel F) and xanthine oxidase (panel G) in response to the identified *Klebsiella* intramammary infection. The sections were cut and mounted on the slides in the histologic “mirror image” perspective in which each feature in the adjacent sections is the exact mirror image of the other precisely in the next focal plane, thus ensuring that what is observed in one cell is, in fact, the same structure in the same cell in the next image. Arrows indicate areas where colocalization of these antigenic signals to epithelial cells was evident.

close proximity generation of the reacting nitrating agent.

The final example of a compartmentalized nitration that affects metabolism is a mitochondrial-based nitration arising during the proinflammatory tissue response to experimental infection of the bovine mammary gland with *E. coli* (Figure 7). In this model, tissues were collected from mammary udder quarters infused with either sterile PBS (control quarters) or 400 cfu *E. coli*

representative of 12 or 24 h of infection. The red “dots” represent immunochemical locations in the cell where events specific to the nitration of the α -subunit of the mitochondrial complex V–ATP-synthase have been located. Figure 7A shows that ATP-synthase nitrations seldom occur in healthy, nonchallenged cells. In stark contrast, the increase in the nitration of this ATP-synthase is >25-fold at 12 and 24 h after infection (Fig. 7, B–D). When these data are subjected to a mathemat-

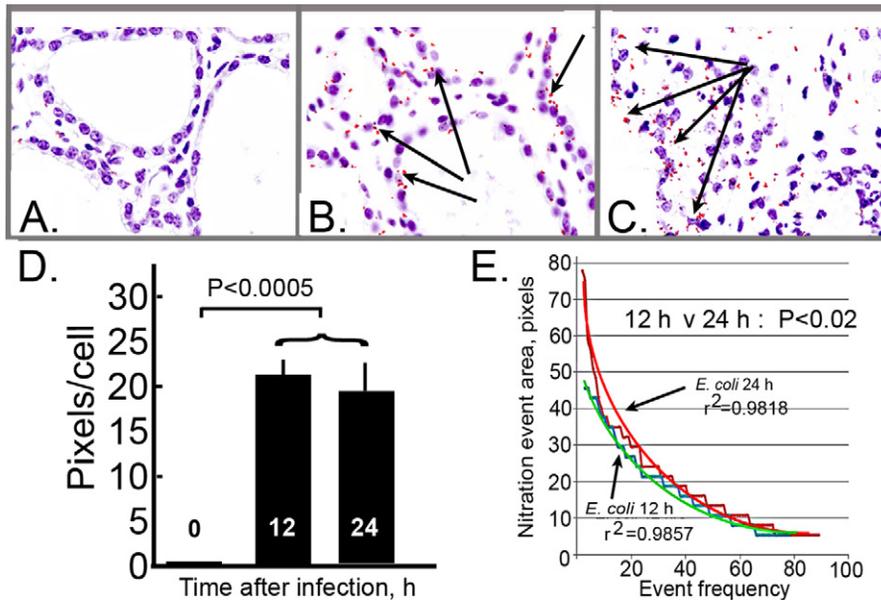


Fig. 7. *Escherichia coli* infection triggers the generation of nitration reactions in specific mitochondrial proteins in mammary epithelial cells. A proximity ligation assay (panel A, control; panel B, *E. coli* 12 h after infection; panel C, 24 h after infection) using one antibody against 3'-nitrotyrosine and another against mitochondrial ATP-synthase α -subunit resolved the specific nitration (> 20-fold increase; panel D) of the ATP-synthase in the mitochondrial compartment (red dots). A curve-fitting analysis (panel E) of the frequency of these nitration events in a cell as a function of their respective intensity (response area pixels) suggests that, with increased time after infection, the magnitude of the affected mitochondrial nitrations is greater. The jagged blue and brown lines are the actual summated data for the data 12 h and 24 h after infection, respectively. The smooth green and red lines are the regression lines fitted (as a logarithmic function) to the respective response time line of events.

ical curve-peeling analysis that fits the size of the nitration events to their frequency of occurrence while the number of events per affected cell remains relatively constant between 12 and 24 h of infection, the frequency and size events increase (Fig. 7E), a phenomenon consistent with the changes in mitochondrial structure known to occur (fission and fusion) during disease stress [48]. Essentially, these images indicate that (1) high-intensity protein nitrations occur in the confines of the mitochondrial membranes during proinflammatory stress and (2) Complex V-ATP-synthase is a target for these nitrations. Preliminary data from our laboratory indicate that these nitrations decrease the efficiency of ATP generation. Cells presenting these specific nitration events are fundamentally energy starved and enter both apoptotic and necrotic pathways to termination.

Collectively, these data strongly imply that the generation of nitrated proteins and probably alterations in function play a part in the disease processes that are harmful to cells during proinflammatory stress. Like so many other cellular processes, as the sensitivity of detection methods is refined, we may learn more about whether the lowest levels of these generated chemical modifications serve as "normal" modulators of cell function and metabolism and where in the process the

breakpoint occurs where the "good" of the process is overwhelmed by the "bad."

Acknowledgements

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