Pyridine nucleotide regulation of hepatic endoplasmic reticulum calcium uptake

Xudong Wang, Gail Mick & Kenneth McCormick

Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama

Keywords
pyridine nucleotides, calcium uptake, microsomes.

Correspondence
Kenneth McCormick, Department of Pediatrics, University of Alabama at Birmingham, CPP M30, 1601 4th Ave South, Birmingham, AL 35233.
Tel: 205-638-9107
Fax: 205-638-9821
E-mail: kmccormick@peds.uab.edu

Funding Information
No funding information provided.

Received: 3 May 2019; Accepted: 31 May 2019
doi: 10.14814/phy2.14151

Physiol Rep, 7 (12), 2019, e14151, https://doi.org/10.14814/phy2.14151

Abstract
Pyridine nucleotides serve an array of intracellular metabolic functions such as, to name a few, shuttling electrons in enzymatic reactions, safeguarding the redox state against reactive oxygen species, cytochrome P450 (CYP) enzyme detoxification pathways and, relevant to this study, the regulation of ion fluxes. In particular, the maintenance of a steep calcium gradient between the cytosol and endoplasmic reticulum (ER), without which apoptosis ensues, is achieved by an elaborate combination of energy–requiring ER membrane pumps and efflux channels. In liver microsomes, net calcium uptake was inhibited by physiological concentrations of NADP. In the presence of 1 mmol/L NADP, calcium uptake was attenuated by nearly 80%, additionally, this inhibitory effect was blunted by concomitant addition of NADPH. No other nicotinamide containing compounds -save a slight inhibition by NAADP-hindered calcium uptake; thus, only oxidized pyridine nucleotides, or related compounds with a phosphate moiety, had an imposing effect. Moreover, the NADP inhibition was evident even after selectively blocking ER calcium efflux channels. Given the fundamental role of endoplasmic calcium homeostasis, it is plausible that changes in cytosolic NADP concentration, for example, during anabolic processes, could regulate net ER calcium uptake.

Introduction
The coupled redox pyridine nucleotides, NAD+/NADH and their phosphorylated congeners NADP+/NADPH, oversee manifold biochemical functions in eukaryotic cells. When reduced, pyridine nucleotides serve as universal electron donors in enzymatic reactions. These ubiquitous cofactors can shuttle two electrons in oxidoreductase reactions, and are fundamental in the anabolic reductive synthesis of fatty acids, phospholipids, steroids, and some amino acids. Other eminent roles for these cofactors include defending against reactive oxidative species, cytochrome P450 enzymatic detoxification, and the modulation of signal transduction.

Insofar as the maintenance of intracellular calcium gradients in several organelles is a requisite for cell viability, it is plausible that pyridine nucleotides also regulate calcium fluxes (Zima et al. 2004; Vangheluwe et al. 2005; Kilfoil et al. 2013; Raffaello et al. 2016). Relative to the cytosol, high ER calcium concentrations are indispensable for cell differentiation and proliferation, the synthesis and chaperone-assisted folding of proteins, activity of oxidoreductases, and possibly gene transcription. Dysregulation of intracellular calcium homeostasis begets ER stress and apoptosis.

In the liver, the endoplasmic reticulum (ER) is the major calcium reservoir, and the concentration therein is regulated by the integration of antithetical fluxes across the membrane. P2-ATPase ion transporters uphold the steep calcium concentrations in the ER. Influx from the cytosol to the ER occurs via a family of P2-ATPase ion transporters, and efflux through discrete ion channels. Considering the ER luminal free calcium concentration of ~400 μmol/L, which is a 1000-fold over that found in the cytosol, this steep gradient consumes considerable ATP energy. A family of membrane anchored sarco/endoplasmic calcium ATPase (SERCA) pumps sustain the
gradient. Serca2b is the isoform present in hepatic microsomes (Anger et al. 1994; Vangheluwe et al. 2005; Vande-caetsbeek et al. 2011).

In addition to a direct pyridine nucleotide effect on calcium channels, their metabolic derivatives may have similar actions. For instance, two derivatives of the nicotinamide NADP, namely, NAADP and ADPR, stimulate calcium efflux from cell organelles, including liver ER (Galione and Ruas 2005; Mandi et al. 2006). Consequently, insofar as NADP inhibits net ER calcium uptake, it is conceivable that the effect could be a result of increased calcium egress, not ingress, resulting from the production of the two aforementioned metabolites.

In vitro experimental approaches must approximate extant eukaryotic pyridine nucleotide concentrations, and their ratios, in disparate tissue organelles. Contingent on the subcellular pool (for example, cytosol, mitochondria, or ER), the phosphorylated or nonphosphorylated pyridine redox ratios can differ logarithmically (>10^4) (Somogyi et al. 2016). And, accordingly, the ratios therein dictate the subcellular metabolic processes in each domain (Hosios and van der Heiden 2018). To illustrate, cytosolic glycolysis and van der Heiden 2018). To illustrate, cytosolic glycolysis

calcium uptake

Calcium uptake was measured in the following medium: 100 mmol/L KCl, 10 mmol/L MOPS buffer, pH 7.2, 5 mmol/L sodium azide, 5 mmol/L MgCl2, 1 mmol/L ATP, 5 mmol/L creatine phosphate, 5 units/mL creatine phosphokinase, 5 mmol/L ammonium oxalate and 100 μmol/L CaCl2 (100 μmol/L free calcium) and 0.1 μCi/mL of 45CaCl2 in a total volume of 0.4 mL. The assay was conducted at 37°C and initiated with the addition of the microsomes to a concentration of 0.1 mg of protein/mL. The reactions were terminated after 30 min by filtering through 0.45 μm membrane filters (Millipore Corp.) and the filters were washed with 0.25 mol/L sucrose (2 mL). The filters were dried and 45Ca2+ was determined using liquid scintillation counter (PerkinElmer) (Moore et al. 1975). Under these standard conditions, namely, 0.1 mg/mL protein and 30 min incubation, the control rate was 5.42 ± 0.19 (SEM) nmol/min/mg protein, similar to those previously reported in liver microsomes. Of note, if ATP is omitted there was no microsomal calcium uptake. The effect of various redox modifiers (at 1 mmol/L) on calcium uptake were tested, including: ascorbate (Vergauwen et al. 2015; Chen and Chang 2018), GSH and GSSG (Jessop and Bulleid 2004; Saaranen et al. 2010; Foyer and Noctor 2011), dehydroascorbate (DHA) (Csala et al. 1999; Saaranen et al. 2010; Foyer and Noctor 2011), H2O2 (Xu et al. 1997; Csala et al. 1999), and dithiothreitol (DTT) (Ushioda et al. 2016).

Materials and Methods

45CaCl2 was purchased from American Radiolabeled Chemicals. D9 (thioredoxin reductase inhibitor) was from Bio-Techne corporation. Other compounds and general chemicals were from Sigma–Aldrich.

Preparation of rat liver microsomes

Microsomes were isolated from the liver of Sprague Dawley rats (150–200 g body weight) as previously described (McCormick et al. 2006; Wang et al. 2011). All animal procedures were undertaken with approval and oversight of the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama Birmingham. Tissue samples were homogenized in an ice-bath with 4 vol. of 0.25 mol/L sucrose and 50 mmol/L Tris/HCl, pH 7.3. The homogenate was centrifuged for 10 min at 1000g. The supernatant portion was removed and centrifuged for 20 min at 10,000g, and, thereafter, centrifuged for 60 min at 100,000g. The resulting pellet was washed twice with the same buffer. The microsomes were resuspended at a protein concentration of 15–20 mg/mL (measured using a Bio-Rad protein assay).

Ca2+ATPase activity

Total ATPase activity (both Mg2+ and Ca2+ in the assay) of liver microsomes was measured with a direct colorimetric assay (Chan et al. 1986). The reaction was initiated by adding 0.1 mg/mL liver microsomes to the assay buffer containing 0.2 mmol/L EGTA, 1 mmol/L Mg2+ATP, 5 mmol/L NaN3, 2.5 μmol/L ruthenium red, and CaCl2 to yield 20 μmol/L free calcium in a buffer of 100 mmol/L...
L KCl and 50 mmol/L imidazole, pH 6.8. In case of any mitochondrial contamination of the microsomal preparation, sodium azide was added to quell calcium uptake by this organelle. After 15 min of incubation at 37°C, 50 μL of this reaction mixture was added to 200 μL assay mix (malachite green), the absorbance of the color complex was measured at 630 nm with a microplate reader (Bio-Rad). Pi quantity was determined from a standard curve prepared with known amounts of KH₂PO₄; the reaction was linear to at least 12.5 nmols Pᵢ. The Mg²⁺-ATPase component was determined by subtracting the control value (no calcium added) from the total ATPase activity.

**Calcium release**

Microsomes were washed once with 6% PEG and diluted to 15 mg/mL with KCl-MOPS buffer containing 100 mmol/L KCl, 20 mmol/L MOPS, 20 mmol/L NaCl, 1 mmol/L MgCl₂, pH 7.2, preloaded with 1 mmol/L CaCl₂, plus 40 μCi/mL ⁴⁵CaCl₂ for 1 h at room temperature. The microsomal suspensions were then diluted 1:100 with the KCl-MOPS buffer to activate the efflux. At different time points, 0.4 mL of suspensions was applied on the 0.45 μm membrane filters and then washed with 3 mL ice-cold buffer containing 0.25 mol/L sucrose, 60 mmol/L Tris-HCl, 1 mmol/L LaCl₃, pH 7.2. The filters were dried and ⁴⁵Ca²⁺ was determined using liquid scintillation counter (Scherer and Deamer 1986; Giunti et al. 2007).

**Statistical analysis**

Data are presented as mean ± SEM (n). The significance of differences between groups was determined using Student’s t test and analysis of variance. A P < 0.05 value was the cutoff level to reject the null hypothesis. All statistical analyses were performed with GraphPad Prism software (San Diego, CA, USA). Mean control values were nominally assigned a value of 100%.

**Results**

Figure 1 depicts the time/microsomal protein relationships with calcium uptake in rat liver microsomes. At 0.5 mg microsomal protein, the uptake was linear for 30 min, and up to 40 min at 0.1 mg protein (data not shown for the 0.1 mg protein). Basal calcium uptake rate was 5.42 ± 0.19 nmol/mg protein/ min, similar to previous reports (Brattin et al. 1982; Erickson et al. 1987; Moore et al. 1975; Thastrup et al. 1990; Mandi et al. 2006). In the absence of ATP, the uptake was minimal.

With NADP concentrations ranging from 0.01 to 2 mmol/L, there was a concentration-dependent reduction in calcium uptake (Fig. 2). At 1 mmol/L NADP, calcium uptake was ~20% of control, and nearly completely quenched at 2 mmol/L. Moreover, the magnitude of the inhibitory action of NADP on calcium uptake was contingent on the free calcium concentration in the assay (Fig. 3). At a free calcium of 500 μmol/L, any inhibitory effect of 1 mmol/L NADP was indiscernible.

Adding NADPH to the assay, thereby changing the pyridine redox state, significantly reversed the uptake inhibition by 1 mM NADP (Fig. 4) – however, even at 5 mmol/L NADPH, calcium uptake did not return to
control values (no NADP). In Figure 5, various compounds containing a nicotinamide moiety were tested. With the exception of NAADP, no other nicotinamide compounds altered calcium uptake. Also, because high concentrations of NAADP desensitize its receptor, both high and low concentrations were also tested: at 1 \text{ mmol/L}, there was no effect on calcium uptake, but at 1 \text{ mmol/L} there was a 28% reduction (Galione 2011). Nicotinamide alone had no consequence.

When microsomes were preloaded with calcium, following which calcium was rapidly removed from the incubation media, there was a rapid outflow of the cation (Fig. 6). There was no difference in the loss of microsomal calcium in the presence/absence of NADP. However, insofar as the calcium uptake methodology requires considerably longer time duration (30 min), studies were also performed in which selective calcium efflux channels were blocked. This set of experiments was conducted to exclude the possibility that, as an alternative explanation, NADP was activating calcium release channels rather than directly stanching ER uptake. Specific channel inhibitors were incubated concomitantly with NADP during the assay (Ozawa 2010). Following channel blockage, the NADP effect on calcium uptake was then determined (Table 1).

Despite blocking ryanodine (RyR), inositol 1, 4, 5 triphosphate (IP$_3$), and NAADP receptors with, respectively, ruthenium red and dantrolene, 2APB, and trans-NED-19, the NADP inhibiting effect on uptake was not attenuated (Mitchell et al. 2003; Galione and Ruas 2005). Unexpectedly, NADP inhibition of uptake in the presence of RyR and IP$_3$ channel blockers was even more distinct. To sum, should NADP have opened an efflux channel, then inhibition of that conduit should have extinguished, or certainly reduced, the inhibitory effect of NADP on calcium uptake; however, with each channel inhibitor, this action was not observed.
Both SERCA and RyR channels can be altered by NOX /NADPH oxidases, especially isoform NOX4 which is abundant in liver endoplasmic reticulum (Laurindo et al. 2014). Reactive oxygen species (ROS) generated by these oxidases are felt to be released in the ER lumen and, therefore, many ER calcium channels could be affected by oxidative/reductive processes (Gorlach et al. 2015). Thus, to explore the possibility that NADP was altering NADPH oxidase activity by adjusting the redox state, a potent NOX4 inhibitor (compound GKT13783) with an IC50 of 10.1 \text{mol/L} was added to the uptake mixture (Fig. 7) (Altenhofer et al. 2015). No effect on the extent of NADP hindrance on uptake was detected.

As aforesaid, ROS compounds can inhibit SERCA and ER calcium channels (Gorlach et al. 2015). In our microsomal assay, oxidizing compounds (H2O2, glutathione disulfide) reduced calcium uptake, whereas dehydroascorbic acid did not (Fig. 8). As for the latter compound, its reduced form, ascorbate, likewise had no uptake effect. The mild inhibition by NADPH alone is unexplained but could be a direct effect or secondary to the generation of NADP via NADPH oxidases present on microsomes. Notably, however, the NADP restraint of uptake was not counteracted by the concomitant addition of reducing agents (DTT and GSH), inferring that ROS are not contributing to the NADP effect.

Insofar as NADP will alter the assay redox balance, a series of experiments were designed to address the possibility of a recently proposed ER-embedded membrane oxidoreductase which is a pivotal component of the flavoprotein thioredoxin reductase 1 (TNR1) sequence (Poet et al. 2017; Ellgaard et al. 2018). Accordingly, a gold (I) compound which is an irreversible inhibitor of TNR1, namely auranofin, was tested (Fig. 9). This compound alone significantly reduced calcium uptake by nearly 70%, yet another potent, more specific, gold (I) inhibitor (called D9) was ineffective. Inasmuch as auranofin can generate ROS, the addition of reducing agents (DTT, GSH) mitigated its inhibition.

Microsomal Ca\textsuperscript{2+}-ATPase activity, as determined by release of phosphate, was inhibited by NADP; however, the magnitude of which did not mirror the results observed in the uptake studies (Fig. 10). At 1 mmol/L NADP, there was a 64% reduction in ATPase activity, appreciably less than the corresponding inhibition on calcium uptake. However, only 14% of the total ATPase activity in liver microsomes is attributable to Ca\textsuperscript{2+}-ATPase, the bulk of the liberated phosphate is derived from Mg\textsuperscript{2+}-ATPase (i.e., thapsigargin insensitive) (de Moel et al. 1995).

### Discussion

The total pyridine nucleotide concentrations used in these experiments were within the physiologic ranges found in assorted tissues. Liver contains submillimolar total(free reduced form, ascorbate, likewise had no uptake effect. The mild inhibition by NADPH alone is unexplained but could be a direct effect or secondary to the generation of NADP via NADPH oxidases present on microsomes. Notably, however, the NADP restraint of uptake was not counteracted by the concomitant addition of reducing agents (DTT and GSH), inferring that ROS are not contributing to the NADP effect.

Insofar as NADP will alter the assay redox balance, a series of experiments were designed to address the possibility of a recently proposed ER-embedded membrane oxidoreductase which is a pivotal component of the flavoprotein thioredoxin reductase 1 (TNR1) sequence (Poet et al. 2017; Ellgaard et al. 2018). Accordingly, a gold (I) compound which is an irreversible inhibitor of TNR1, namely auranofin, was tested (Fig. 9). This compound alone significantly reduced calcium uptake by nearly 70%, yet another potent, more specific, gold (I) inhibitor (called D9) was ineffective. Inasmuch as auranofin can generate ROS, the addition of reducing agents (DTT, GSH) mitigated its inhibition.

Microsomal Ca\textsuperscript{2+}-ATPase activity, as determined by release of phosphate, was inhibited by NADP; however, the magnitude of which did not mirror the results observed in the uptake studies (Fig. 10). At 1 mmol/L NADP, there was a 64% reduction in ATPase activity, appreciably less than the corresponding inhibition on calcium uptake. However, only 14% of the total ATPase activity in liver microsomes is attributable to Ca\textsuperscript{2+}-ATPase, the bulk of the liberated phosphate is derived from Mg\textsuperscript{2+}-ATPase (i.e., thapsigargin insensitive) (de Moel et al. 1995).

### Table 1. Effect of various calcium channel blockers, with and without NADP, on Ca\textsuperscript{2+} uptake in rat liver microsomes

| Ca\textsuperscript{2+} channels | Inhibitors (10 \textmu mol/L) | % control |
|-------------------------------|-------------------------------|-----------|
|                               | Inhibitor alone | Inhibitor plus 0.5 mmol/L NADP | 0.5 mmol/L NADP alone |
| NAADP                         | trans-Ned-19 90.7 ± 0.7 | 65.6 ± 0.8 | 61.8 ± 3.3 |
| IP3                           | 2APB 93.2 ± 1.8 | 44.2 ± 3.1 | 61.8 ± 3.3 |
| RyR                           | RR 95.0 ± 2.2 | 43.9 ± 4.8 | 61.8 ± 3.3 |
|                               | Dantrolene 95.7 ± 2.3 | 44.7 ± 4.7 | 61.8 ± 3.3 |

Microsomes were incubated for 5 min with trans-Ned-19, RR, 2APB or dantrolene at 10 \textmu mol/L alone or in combination with 0.5 mmol/L NADP. Thereafter, calcium uptake was determined as described in the Materials and Methods section. Results are expressed as mean ± SEM.

None of the channel inhibitors alone had a significant (P > 0.05) effect on uptake. NAADP, Nicotinic acid adenine dinucleotide phosphate; 2APB: 2-Aminoethoxydiphenyl borate; RR, Ruthenium red.
and bound) concentrations of pyridine nucleotides. For example, in hepatocytes, assuming approximately 2 mL of water per g dry weight, the total cellular concentration of NADP$^+$ and NADPH in the cell is ~0.9 mmol/L (Tischler et al. 1977). As pertains to the corresponding nonphosphorylated nucleotides (NAD$^+$ and NADH), the total cellular concentration is ~1.5 mmol/L. In liver, the cytosolic NADP concentrations are ~0.35 mmol/L based on 173 nmol/g wet weight and 53% of wet weight is
intracellular water (Lindall and Lazarow 1964; Cieslar et al. 1998).

Both the cytosolic and ER redox states can modulate not only ER calcium exit channels, but also uptake via transporter calcium ATPases (e.g. SERCA2b) (Morris and Sulakhe 1997; Xu et al. 1997; Li and Camacho 2004; Zima et al. 2004; Gorlach et al. 2006; Ozawa 2010; Gorlach et al. 2015; Ushioda et al. 2016). Oxidation of protein sulfhydryl groups diminishes Ca$^{2+}$-ATPase activity (Horakova et al. 2013). Several pyridine containing compounds were tested, with or without the antioxidants GSH and DTT (Fig. 8). Not unexpectedly, oxidizing agents (H$_2$O$_2$ or GSSG) attenuated calcium uptake (Scherer and Deamer 1986; Morris and Sulakhe 1997; Xu et al. 1997; Hidalgo et al. 2005; Ozawa 2010; Gorlach et al. 2015; Ushioda et al. 2016). Yet the concomitant addition of reducing compounds (GSH or DTT) failed to prevent the NADP restriction of uptake- this infers that the nucleotide effect on uptake was extraneous to the generation of ROS, thereby inhibiting SERCA. In short, none of the inhibitors alone significantly inhibited or stimulated calcium uptake, nor did they change the NADP inhibition.

The uptake inhibition due to NADP was apparent even in the presence of specific inhibitors of calcium release channels, such as IP$_3$ and RyR (but, arguably, liver ER membranes may be devoid of RyR) (Shoshan-Barmatz 1990; Lilly and Gollan 1995; Mitchell et al. 2003; Hidalgo et al. 2004, 2005; Giunti et al. 2007; Saleem et al. 2014). This data infers that calcium influx is the site(s) of net uptake inhibition by NADP.

When NADPH was added to adjust the redox ratio, the NADP inhibition was blunted. The relevance of a redox ratio (NADH/NAD) was likewise demonstrated in permeabilized ventricular myocytes by assessing ER calcium egress via the RyR conduit (Zima et al. 2004). Our experiments corroborated the relevance of the pyridine nucleotide redox ratio affecting net ER calcium flux.

Another second messenger that mobilizes stored calcium, especially from acidic vesicles such as lysosomes, is nicotinic acid adenine dinucleotide phosphate (NAADP) (Galione 2011). This compound is a derivative of NADP, generated by ADP-ribozyme cyclase (CD 38), abundant in plasma membranes but also found, but to a lesser degree, in nonskeletal microsomes (Chini et al. 2002; Meszaros and Bak 1992). In the presence of nicotinic acid and low pH, the enzyme converts NADP to NAADP and nicotinamide-in sum, a carboxyl moiety has replaced the NADP amide. Of note, in one relevant study, NADP had no effect on calcium release from sarcoplasmic reticulum (Hohenegger et al. 2002). There was a slight reduction (28%) in uptake at 1 mmol/L NAADP, but this did not modify the inhibitory action of NADP when the two compounds were combined. At 1 mmol/L concentration, NAADP reduced the net uptake by 28%; inhibition was not observed at 1 μmol/L. And although the inhibition by NADP is mitigated by NADPH, this was not the case with NAADP- namely, 1 mmol/L NADPH did not counter the 25–30% inhibition due to NAADP (Fig. 5). Nicotinamide (1 mmol/L) alone did not alter calcium uptake. As a final point, it should be noted that the inhibition by NADP was not secondary to the generation of NAADP. Although this latter nucleotide can be catalytically derived from NADP by a base-exchange reaction via ADP-riboyl cyclase, in which nicotinic acid is the substrate, the presence of both (enzyme and substrate) in liver microsomal preparation is highly improbable.

Two previous studies concluded that NADPH>NADP slightly inhibited calcium uptake in hepatic microsomes (Prasad et al. 1986; Erickson et al. 1987). In these experiments, NADPH was not directly added to the assay, rather it was generated via the oxidation of G6P by the inclusion of G6P dehydrogenase. This would, in turn, lower the concentration of G6P in the assay, a phosphorlated compound which detains calcium in the ER lumen (possibly by liberating phosphate, forming insoluble salts to thwart backflow) (Wolf et al. 1986; Romani et al. 1988; Fulceri et al. 1990; van Schaftingen and Gerin 2002). Because the ER membrane is permeable to oxalate, this anion serves a calcium-impounding role in nearly all microsomal uptake experiments. Nevertheless, in terms of net calcium sequestration in microsomes, the two compounds (oxalate/G6P) together in our uptake assay are additive (data not shown). Consequently, by consuming G6P to synthesize NADPH, this leads to an attenuation of calcium uptake, not by a direct inhibition of microsomal uptake by NADPH, but rather by decreasing the G6P capacity to sequester ER lumenal calcium. This can lead to a specious conclusion that NADPH per se inhibits calcium uptake.

Intriguingly, both reductive and oxidizing processes coexist in the ER (Margittai et al. 2015). Inasmuch as the ER lumen is predominantly an oxidized thio-disulfide milieu, precisely how reducing electrons can coexist in this setting is unresolved. For example, a reduction process in the ER involves the conversion of cortisone (inactive) to cortisol (active) via the bidirectional enzyme.11-β hydroxysteroid dehydrogenase. And to correct the nosious disulfide bonds of misfolded proteins in the ER, protein disulfide isomerases require electrons from elements in the cytosol (Poet et al. 2017; Ellgaard et al. 2018). Recent evidence suggests that these cytosolic electrons, derived ultimately from the nutrient G6P, are delivered to the ER lumen via cytosolic thioredoxin reductase 1 (TNR1) and NADPH (Dagnell et al. 2017; Poet et al. 2017; Ellgaard et al. 2018). To accomplish this task, reducing equivalents are ultimately shuttled across the ER.
membrane by a yet unidentified membrane-embedded oxidoreductase (Poet et al. 2017). Therefore, experiments were designed to address the possibility that the cytosolic thioredoxin reductase 1 pathway, by changing the ER redox state, could impact ion fluxes. Admittedly, under the experimental conditions, the existence of aTNR1 pathway is unlikely insofar as isolated microsomes are devoid of soluble cytosolic enzymes. Although auranofin per se inhibited calcium uptake, a more specific inhibitor of TNR1 (termed D9) was ineffectual (Saccoccia et al. 2014; Zhang et al. 2014). Hence, calcium uptake inhibition by NADP is not acting through a thioredoxin cascade, and the effect of auranofin in all likelihood is secondary to its capacious and potent inhibition of flavoprotein pyridine oxidoreductases or through ROS production (Vint et al. 1994; Saccoccia et al. 2014; Varghese and Busselberg 2014; Wang et al. 2017). The latter likelihood is supported by the observation that reducing agents (GSH and DTT) tempered the inhibition from auranofin (Fig. 8).

Besides the TNR1 system, there exist at least seven selenoproteins in the ER, several of which modulate calcium flux (Appenzeller-Herzog and Simmen 2016; Addinsall et al. 2018). Auranofin, acting nonspecifically, conceivably modifies several of these selenium-containing flavoprotein oxidoreductases (Saccoccia et al. 2014). We found a 64% inhibition of calcium uptake by auranofin.

Figure 11. Schematic of the proposed nutritional regulation of pyridine nucleotide-mediated endoplasmic reticulum calcium uptake. (A) In the well-fed state, the cellular influx of carbohydrates and calories leads to an increase in the cytosolic NADP/NADPH ratio. This in turn inhibits endoplasmic reticulum calcium uptake. (B) In the fasting state, diminished cellular carbohydrate and calorie influx reduces the nutritional inhibition of calcium uptake. RyR, ryanodine receptor; IP₃R, inositol 1, 4, 5–triphosphate receptor.
Nevertheless, auranofin, by binding irreversibly to selenol motifs in enzymes, is promiscuously nonspecific, and may directly interact to unfavorably reduce activity of an array of diverse non selenoenzymes (e.g. adenylyl cyclase), or through the production of ROS (Lazarevic et al. 1992; Vint et al. 1994; Wang et al. 2017).

Given that ROS can modify ER release channels, as well as SERCA, a change in the pyridine nucleotide redox ratio could, in turn, regulate NOX NADPH oxidases present in the ER (Laurindo et al. 2014). To ensure that ROS were not generated from NOX NADPH oxidases, in particular, the NOX 4 isoform associated with ER, specific enzyme inhibitors were added to the uptake studies (Altenhofer et al. 2015). Suppressing NOX activity did not modify the NADP effect on calcium uptake, nor did the concomitant addition of compounds which can curb ROS oxidation (Table 1).

Given the low free calcium concentrations in the uptake assay, and the dependence of the % NADP inhibition on free calcium, any physiochemical sequestering of calcium by binding to other compounds should be considered. To wit, a calcium-pyridine nucleotide complex could lower the free calcium concentration in the assay, thereby reducing uptake, and resulting in a spurious conclusion. As it turns out, the association constant for NADPH is twofold greater than NADP; hence, NADPH would be expected to be speciously more “inhibitory” in our uptake assay if a reduction in free calcium was responsible (Burkhard 1982).

In conclusion, NADP inhibits calcium uptake in hepatic microsomes and the site of action ostensibly involves the ingress component of net calcium uptake (Kilfoil et al. 2013). Many classes of ion channels are modulated by pyridine nucleotides and, of note, even fat synthesis can be attenuated by NADP acting directly on fatty acid synthetase (Erickson et al. 1987; Stern and Smith 1987; Tipparanju et al. 2005; Tamsett et al. 2009). Seemingly, for ER calcium uptake inhibition, the phosphate moiety of the pyridine nucleotide is a requisite insofar as neither NAD, nicotinamide, nor NAAD were inhibitory in our assay (Agledal et al. 2010). Presently unknown is whether NADP inhibition involves a cofactor mass action effect on an embedded ER oxidoreductase or, alternatively, that this nucleotide binds to a regulatory site within an ingress channel.

Lastly, as pertains to pathology, disturbed lipid and pyridine nucleotide metabolism, commonly encountered in obesity, impairs endoplasmic calcium uptake which, in so doing, begets ER stress (Luciani et al. 2009; Park et al. 2010; Fu et al. 2011). And, with obesity in mind, the amount of nutrient intake adjusts the intracellular redox state (Fig. 11). Case in point, in rodents that are well fed with a high sucrose diet, versus those that are fasting, the hepatic free (unbound) NADP/ NADPH ratio in the cytosol increases nearly fivefold to eightfold (Veech et al. 1969; Tischler et al. 1977; Laurindo et al. 2014). Hence, it is not inconceivable that the cytosolic accumulation of NADP, due to excess caloric intake, could have similar consequences on ER calcium uptake and stress as does disturbed lipid metabolism.

**Conflict of Interest**

None declared.

**References**

Addinsall, A. B., C. R. Wright, S. Andrikopoulos, C. van der Poel, and N. Stupka. 2018. Emerging roles of endoplasmic reticulum-resident selenoproteins in the regulation of cellular stress responses and the implications for metabolic disease. Biochim Biophys Acta 1862:774–87.

Agledal, L., M. Niere, and M. Ziegler. 2010. The phosphate makes a difference: cellular functions of NADP. Redox Rep. 15:2–10.

Altenhofer, S., K. A. Radermacher, P. W. Kleikers, K. Wingler, and H. H. Schmidt. 2015. Evolution of NADPH oxidase inhibitors: selectivity and mechanisms for target engagement. Antioxid. Redox Signal. 23:406–427.

Anger, M., J. L. Samuel, F. Marotte, F. Wuytack, L. Rappaport, and A. M. Lompre. 1994. In situ mRNA distribution of sarco(endo)plasmic reticulum Ca(2+)-ATPase isoforms during ontogeny in the rat. J. Mol. Cell. Cardiol. 26:539–550.

Appenzeller-Herzog, C., and T. Simmen. 2016. ER-luminal thiol/selenol-mediated regulation of Ca2+ signalling. Biochem. Soc. Trans. 44:452–459.

Brattin, W. J. Jr, R. L. Waller, and R. O. Recknagel. 1982. Analysis of microsomal calcium sequestration by steady state isotope exchange. Enzyme kinetics and role of membrane permeability. J. Biol. Chem. 257:10044–10051.

Burkhard, R. K. 1982. Interactions of calcium with the pyridine nucleotides. Arch. Biochem. Biophys. 218:207–212.

Chan, K. M., D. Delfert, and K. D. Junger. 1986. A direct colorimetric assay for Ca2+-stimulated ATPase activity. Anal. Biochem. 157:375–380.

Chen, G., and T. M. S. Chang. 2018. Dual effects include antioxidant and pro-oxidation of ascorbic acid on the redox properties of bovine hemoglobin. Artif. Cells Nanomed. Biotechnol. 46:983–992.

Chini, E. N., C. C. Chini, I. Kato, S. Takasawa, and H. Okamoto. 2002. CD38 is the major enzyme responsible for synthesis of nicotinic acid-adenine dinucleotide phosphate in mammalian tissues. Biochem. J. 362:125–130.

Cieslar, J., M. T. Huang, and G. P. Dobson. 1998. Tissue spaces in rat heart, liver, and skeletal muscle in vivo. Am. J. Physiol. 275:R1530–R1536.
Cscala, M., L. Braun, V. Mile, T. Kardon, A. Szarka, P. Kupcsulik, et al. 1999. Ascorbate-mediated electron transfer in protein thiol oxidation in the endoplasmic reticulum. FEBS Lett. 460:539–543.

Dagnell, M., P. E. Pace, Q. Cheng, J. Frijhoff, A. Ostman, E. S. J. Arner, et al. 2017. Thioredoxin reductase 1 and NADPH directly protect protein tyrosine phosphatase 1B from inactivation during H₂O₂ exposure. J. Biol. Chem. 292:14371–14380.

Ellgaard, L., C. S. Sevier, and N. J. Bulleid. 2018. How are proteins reduced in the endoplasmic reticulum? Trends Biochem. Sci. 43:32–43.

Ercksson, R. R., J. S. Prasad, and J. L. Holtzman. 1987. The role of NADPH- and reduced glutathione-dependent enzymes in the norepinephrine modulation of the ATP-dependent, hepatic microosomal calcium pump: a new pathway for the noradrenergic regulation of cytosolic calcium in the hepatocyte. J. Pharmacol. Exp. Ther. 242:472–477.

Foyer, C. H., and G. Noctor. 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 155:292–301.

Gorlach, A., K. Bertram, S. Hudecova, and O. Krizanova. 2015. Calcium homeostasis and sources of hydrogen peroxide. Redox Biol. 3:340–347.

Gorlach, A., P. Klappa, and T. Kietzmann. 2006. The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxid. Redox Signal. 8:1391–1418.

Hidalgo, C., R. Bull, M. I. Behrens, and P. Donoso. 2004. Redox regulation of RyR-mediated Ca²⁺ release in muscle and neurons. Biol. Res. 37:539–552.

Hidalgo, C., P. Donoso, and M. A. Carrasco. 2005. The ryanodine receptors Ca²⁺ release channels: cellular redox sensors? IUBMB Life 57:315–322.

Hohenegger, M., J. Suko, R. Gescheidinger, H. Drobny, and A. Zidar. 2002. Nicotinic acid-dinucleotide phosphate activates the skeletal muscle ryanodine receptor. Biochem. J. 367:423–431.

Horakova, L., M. K. Strosova, C. M. Spickett, and D. Blaskovic. 2013. Impairment of calcium ATPases by high glucose and potential pharmacological protection. Free Radic. Res. 47(Suppl 1):81–92.

Hosios, A. M., and M. G. van der Heiden. 2018. The redox requirements of proliferating mammalian cells. J. Biol. Chem. 293:7490–7498.

Jessop, C. E., and N. J. Bulleid. 2004. Glutathione directly reduces an oxidoreductase in the endoplasmic reticulum of mammalian cells. J. Biol. Chem. 279:55341–55347.

Killooil, P. J., S. M. Tipparaju, O. A. Barski, and A. Bhattacharjee. 2013. Regulation of ion channels by pyridine nucleotides. Circ. Res. 112:721–741.

Laurindo, F. R., T. L. Araujo, and T. B. Abrahao. 2014. NADPH oxidases and the endoplasmic reticulum. Antioxid. Redox Signal. 20:2755–2775.

Lazarevic, M. B., K. Yan, W. I. Swedler, M. M. Rasenick, and J. L. Skosey. 1992. Effect of gold compounds on the activity of adenyl cyclase in human lymphocyte membranes. Arthritis Rheum. 35:857–864.

Li, Y., and P. Camacho. 2004. Ca²⁺-dependent redox modulation of SERCA 2b by ERp57. J. Cell Biol. 164:35–46.

Lilly, L. B., and J. L. Gollan. 1995. Ryanodine-induced calcium release from hepatic microsomes and permeabilized hepatocytes. Am. J. Physiol. 268:G1017–G1024.

Lindall, A. W. Jr, and A. Lazarow. 1964. A critical study of pyridine nucleotide concentrations in normal fed, normal fasted, and diabetic rat liver. Metabolism 13:259–271.

Luciani, D. S., K. S. Gwiazda, T. L. Yang, T. B. Kalynyak, Y. Bychkivska, M. H. Frey, et al. 2009. Roles of IP3R and RyR Ca²⁺ channels in endoplasmic reticulum stress and beta-cell death. Diabetes 58:422–432.

Mandi, M., B. Toth, G. Timar, and J. Bak. 2006. Ca²⁺ release triggered by NADPH in hepatocyte microsomes. Biochem. J. 393:235–238.

Margittai, E., B. Enyedi, M. Cscala, M. Geiszt, and G. Banhegyi. 2015. Composition of the redox environment of the endoplasmic reticulum and sources of hydrogen peroxide. Free Radic. Biol. Med. 83:331–340.

McCormick, K. L., X. Wang, and G. J. Mick. 2006. Evidence that the 11 beta-hydroxysteroid dehydrogenase (11 beta-HSD1) is regulated by pentose pathway flux. Studies in rat adipocytes and microsomes. J. Biol. Chem. 281:341–347.

Meszaros, L. G., and J. Bak. 1992. Simultaneous internalization and binding of calcium during the initial phase of calcium uptake by the sarcoplasmic reticulum Ca pump. Biochemistry 31:1195–1200.

Mitchell, K. J., F. A. Lai, and G. A. Rutter. 2003. Ryanodine receptor type 1 and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca²⁺ release from insulin-
containing vesicles in living pancreatic beta-cells (MIN6). J. Biol. Chem. 278:11057–11064.
de Moel, M. P., F. H. van de Put, T. M. Vermegen, J. H. de Pont, and P. H. Willems. 1995. Effect of the aminosteroid, U73122, on Ca2+ uptake and release properties of rat liver microsomes. Eur. J. Biochem. 234:626–631.
Moore, L., T. Chen, H. R. Jr Knapp, and E. J. Landon. 1975. Energy-dependent calcium sequestration activity in rat liver microsomes. J. Biol. Chem. 250:4562–4568.
Morris, T. E., and P. V. Sulakhe. 1997. Sarcoplasmic reticulum Ca(2+)-pump dysfunction in rat cardiomyocytes briefly exposed to hydroxyl radicals. Free Radic. Biol. Med. 22:37–47.
Ozawa, T. 2010. Modulation of ryanodine receptor Ca2+ channels (Review). Mol. Med. Rep. 3:199–204.
Park, S. W., Y. Zhou, J. Lee, J. Lee, and U. Ozcan. 2010. Sarco (endo)plasmic reticulum Ca2+-ATPase 2b is a major regulator of endoplasmic reticulum stress and glucose homeostasis in obesity. Proc. Natl Acad. Sci. USA 107:19320–19325.
Poet, G. J., O. B. Oka, M. van Lith, Z. Cao, P. J. Robinson, M. A. Pringle, et al. 2017. Cytosolic thioredoxin reductase 1 is required for correct disulﬁde formation in the ER. EMBO J. 36:693–702.
Prasad, J. S., R. R. Erickson, D. L. Crankshaw, and J. L. Holtzman. 1986. The effect of cytochrome P-450 and reduced glutathione on the ATP-dependent calcium pump of hepatic microsomes from male rats. Arch. Biochem. Biophys. 248:639–645.
Raffaello, A., C. Mammucari, G. Gherardi, and R. Rizzuto. 2016. Calcium at the center of cell signaling: interplay between endoplasmic reticulum, mitochondria, and lysosomes. Trends Biochem. Sci. 41:1035–1049.
Romani, A., R. Fulceri, A. Pompeia, and A. Benedetti. 1988. MgATP-dependent, glucose 6-phosphate-stimulated liver microsomal Ca2+ accumulation: difference between rough and smooth microsomes. Arch. Biochem. Biophys. 266:1–9.
Saaranen, M. J., A. R. Karala, A. K. Lappi, and L. W. Ruddock. 2010. The role of dehydroascorbate in disulﬁde bond formation. Antioxid. Redox Signal. 12:15–25.
Raccoccia, F., F. Angelucci, G. Boumis, D. Carotti, G. Desiato, A. E. Miele, et al. 2014. Thioredoxin reductase and its inhibitors. Curr. Protein Pept. Sci. 15:621–646.
Saleem, H., S. C. Tovey, T. F. Molinski, and C. W. Taylor. 2014. Interactions of antagonists with subtypes of inositol 1,4,5-trisphosphate (IP3) receptor. Br. J. Pharmacol. 171:3298–3312.
van Schaftingen, E., and I. Gerin. 2002. The glucose-6-phosphatase system. Biochem. J. 362:513–532.
Scherer, N. M., and D. W. Deamer. 1986. Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca2+-ATPase. Arch. Biochem. Biophys. 246:589–601.
Shoshan-Barmatz, V. 1990. High affinity ryanodine binding sites in rat liver endoplasmic reticulum. FEBS Lett. 263:317–320.
Somogyi, A., G. Horvai, M. Csala, and B. Toth. 2016. Analytical approaches for the quantitation of redox-active pyridine dinucleotides in biological matrices. Period Polytech. Chem. Eng. 60:218–230.
Sterng, A., and S. Smith. 1987. Inhibition of mammalian fatty acid synthetase activity by NADP involves decreased mobility of the 4’-phosphopantetheine prosthetic group. J. Biol. Chem. 262:5087–5092.
Tamett, T. J., K. E. Picchione, and A. Bhattacharjee. 2009. NAD+ activates KNa channels in dorsal root ganglion neurons. J. Neurosci. 29:5127–5134.
Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. Proc. Natl Acad. Sci. USA 87:2466–2470.
Tipparaju, S. M., N. Saxena, S. Q. Liu, R. Kumar, and A. Bhattachagar. 2005. Differential regulation of voltage-gated K+ channels by oxidized and reduced pyridine nucleotide coenzymes. Am. J. Physiol. Cell Physiol. 288:C366–C376.
Tischler, M. E., D. Friedrichs, K. Coll, and J. R. Williamson. 1977. Pyridine nucleotide distributions and enzyme mass action ratios in hepatocytes from fed and starved rats. Arch. Biochem. Biophys. 184:222–236.
Ushioda, R., A. Miyamoto, M. Inoue, S. Watanabe, M. Okumura, K. I. Maegawa, et al. 2016. Redox-assisted regulation of Ca2+ homeostasis in the endoplasmic reticulum by disulﬁde reductase ERdj5. Proc. Natl Acad. Sci. USA 113:E6055–E6063.
Vandecaeptbeek, L., P. Vangheluwe, L. Raeymaekers, F. Wuytack, and J. Vanoeveren. 2011. The Ca2+ pumps of the endoplasmic reticulum and Golgi apparatus. Cold Spring Harb. Perspect. Biol. 3:004184.
Vangheluwe, P., L. Raeymaekers, L. Dode, and F. Wuytack. 2005. Modulating sarco(endo)plasmic reticulum Ca2+ ATPase 2 (SERCA2) activity: cell biological implications. Cell Calcium 38:291–302.
Varghese, E., and D. Busselberg. 2014. Auranofin, an anti-rheumatic gold compound, modulates apoptosis by elevating the intracellular calcium concentration ([Ca2+]i) in mcf-7 breast cancer cells. Cancers (Basel) 6:2243–2258.
Veech, R. L., L. V. Eggleston, and H. A. Krebs. 1969. The redox state of free nicotinamide-adenine dinucleotide phosphate in the cytoplasm of rat liver. Biochem. J. 115:609–619.
Vergauwen, H., B. Tambuyser, K. Jennes, J. Degroote, W. Wang, S. de Smet, et al. 2015. Trolox and ascorbic acid reduce direct and indirect oxidative stress in the IPEC-J2 cells, an in vitro model for the porcine gastrointestinal tract. PLoS ONE 10:e0120485.
Vint, I. A., J. C. Foreman, and B. M. Chain. 1994. The gold anti-rheumatic drug auranofin governs T cell activation by enhancing oxygen free radical production. Eur. J. Immunol. 24:1961–1965.

Wang, X., G. J. Mick, E. Maser, and K. McCormick. 2011. Manifold effects of palmitoylcarnitine on endoplasmic reticulum metabolism: 11beta-hydroxysteroid dehydrogenase 1, flux through hexose-6-phosphate dehydrogenase and NADPH concentration. Biochem. J. 437:109–115.

Wang, H., S. Bouzakoura, S. de Mey, H. Jiang, K. Law, I. Dufait, et al. 2017. Auranofin radiosensitizes tumor cells through targeting thioredoxin reductase and resulting overproduction of reactive oxygen species. Oncotarget 8:35728–35742.

Wolf, B. A., J. R. Colca, P. G. Comens, J. Turk, and M. L. McDaniel. 1986. Glucose 6-phosphate regulates Ca2+ steady state in endoplasmic reticulum of islets. A possible link in glucose-induced insulin secretion. J. Biol. Chem. 261:16284–16287.

Xu, K. Y., J. L. Zweier, and L. C. Becker. 1997. Hydroxyl radical inhibits sarcoplasmic reticulum Ca(2+)-ATPase function by direct attack on the ATP binding site. Circ. Res. 80:76–81.

Zhang, D., Z. Xu, J. Yuan, Y. X. Zhao, Z. Y. Qiao, Y. J. Gao, et al. 2014. Synthesis and molecular recognition studies on small-molecule inhibitors for thioredoxin reductase. J. Med. Chem. 57:8132–8139.

Zima, A. V., J. A. Copello, and L. A. Blatter. 2004. Effects of cytosolic NADH/NAD(+) levels on sarcoplasmic reticulum Ca(2+) release in permeabilized rat ventricular myocytes. J. Physiol. 555:727–741.