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Hydrogen peroxide-mediated oxidative stress disrupts calcium binding on calmodulin: More evidence for oxidative stress in vitiligo

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Abstract

Patients with acute vitiligo have low epidermal catalase expression/activities and accumulate 10^{-3} M H_2O_2. One consequence of this severe oxidative stress is an altered calcium homeostasis in epidermal keratinocytes and melanocytes. Here, we show decreased epidermal calmodulin expression in acute vitiligo. Since 10^{-3} M H_2O_2 oxidises methionine and tryptophan residues in proteins, we examined calcium binding to calmodulin in the presence and absence of H_2O_2 utilising ^{45}calcium. The results showed that all four calcium atoms exchanged per molecule of calmodulin. Since oxidised calmodulin looses its ability to activate calcium ATPase, enzyme activities were followed in full skin biopsies from lesional skin of patients with acute vitiligo ($n = 6$) and healthy controls ($n = 6$). The results yielded a 4-fold decrease of ATPase activities in the patients. Computer simulation of native and oxidised calmodulin confirmed the loss of all four calcium ions from their specific EF-hand domains. Taken together H_2O_2-mediated oxidation affects calcium binding in calmodulin leading to perturbed calcium homeostasis and perturbed L-phenylalanine-uptake in the epidermis of acute vitiligo.

Keywords: Calcium; Calmodulin; Hydrogen peroxide; Vitiligo

One hallmark of the depigmentation disorder vitiligo is a loss of functioning melanocytes in the depigmented epidermis [1]. The pathogenesis of this ancient disfiguring disease is still unknown. Over the last decade there is increasing evidence that H_2O_2-mediated oxidative stress is a major player in the onset as well as in the progression of vitiligo. In fact concentrations of H_2O_2 in the mM range have been demonstrated by *in vivo* Fourier Transform-Raman spectroscopy [2,3]. Both the epidermis and to a lesser extent even the vascular system of patients with active vitiligo have been shown to be affected by the constant accumulation of H_2O_2 leading to a cascade of impaired signals [2–12]. Moreover, earlier studies showed also an impaired uptake and efflux of calcium in both epidermal keratinocytes and melanocytes established from lesional skin of patients with vitiligo compared to healthy controls [13,14]. However, the precise mechanism behind this observation escaped definition so far. In this context, it is noteworthy that the calcium binding protein calmodulin is subject to oxidative stress consequently affecting calcium dependent ATPase activities which in turn controls the transport/uptake of the essential amino acid L-phenylalanine [15,16]. This transport as well as its turnover to L-tyrosine via phenylalanine hydroxylase (PAH, EC 1.14.16.1) has been shown to be impaired in patients with vitiligo [16]. Taking into consideration that L-phenylalanine is coupled to the release/uptake of calcium into the cytosol of melanocytes to initiate melanogenesis [17], we have readdressed this issue under conditions of H_2O_2-mediated oxidative stress using the model disease vitiligo.

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In situ immunofluorescence studies showed that calmodulin expression is significantly decreased in the lesional skin of patients with acute vitiligo returning to normal levels after reduction of epidermal H_2O_2 with pseudocatalase PC-KUS. These results suggested that the expression of the protein could be affected by epidermal H_2O_2. In order to explore this possibility in more detail, we tested the binding of calcium on calmodulin utilising the isotope ^{45}calcium on native and H_2O_2 oxidised calmodulin. The results showed that all four calcium atoms dissociated from the protein after oxidation. Structural modelling based on the X-ray structure of calmodulin confirmed severe alteration of all four EF-hand binding sites. Consecutive measurements of calcium ATPase activities in 3 mm skin biopsies obtained from the lesional skin of patients with acute vitiligo compared to healthy controls yielded a 4-fold loss in activity compared to controls. We provide evidence that the calmodulin regulated calcium homeostasis is altered by H_2O_2-mediated stress due to the loss of calcium binding on the protein which in turn affects its functionality. Using the model disease vitiligo for oxidative stress, we were able to show that low epidermal calmodulin levels can be reversed by pseudocatalase PC-KUS. These novel data add yet another target to the list of affected mechanisms in the depigmentation disorder vitiligo. Moreover, the effect of H_2O_2-mediated oxidation on all four EF-hand binding sites offers insight into a new general regulation by calmodulin.

Materials and methods

Materials. Radiolabelled $^{45}CaCl_2$ (12.2 mCi/mg) was obtained from MPI. Calmodulin, pyruvate kinase, lactic dehydrogenase, A23187, Sephadex G-25, NADPH were obtained from Sigma (Poole, Dorset, UK). Phosphenol pyruvate (PEP) was obtained from Boehringer Mannheim (Mannheim, Germany).

Human tissue samples. Full skin 3 mm biopsies were obtained under local anesthesia from the lesional skin of six patients with acute vitiligo and from six healthy age matched controls. All subjects had skin phototype III (Fitzpatrick classification) [18]. The study was approved by the local ethics committee and conducted after signed consent of each participant in accordance with the guidelines in the Declaration of Helsinki Principles.

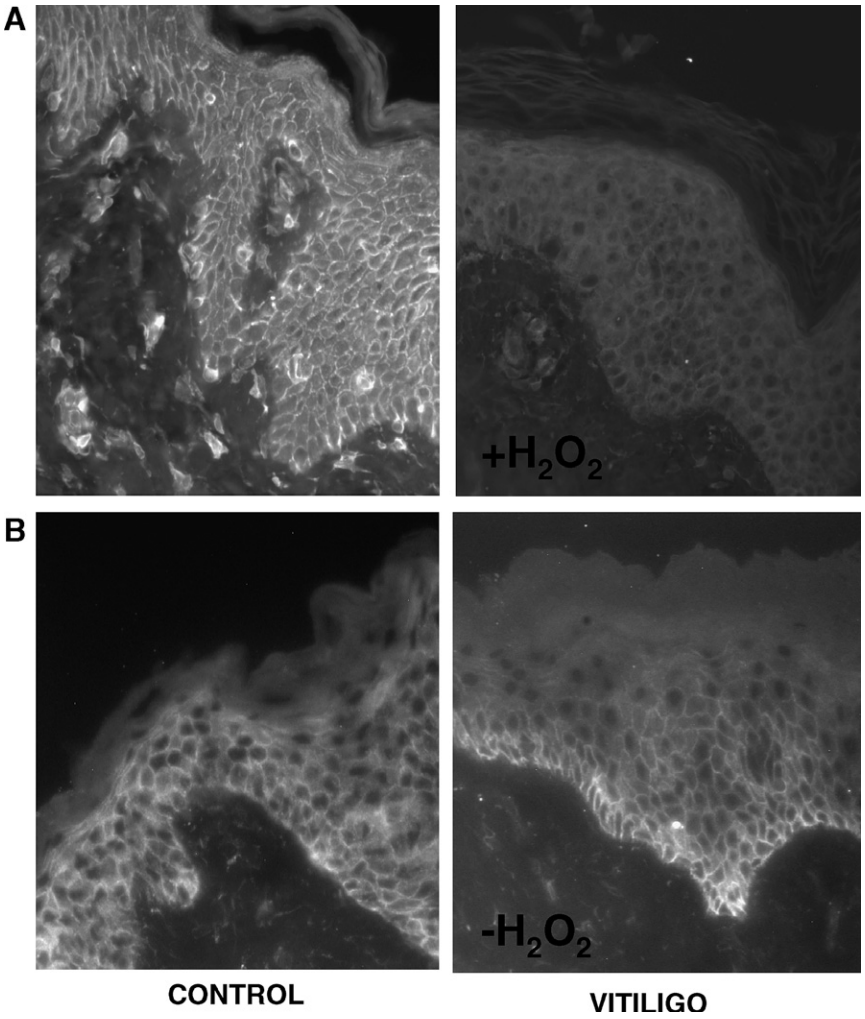

Fig. 1. Decreased *in situ* expression of calmodulin in acute vitiligo. (A) Decreased epidermal calmodulin immune reactivity in active vitiligo ($+H_2O_2$). (B) After reduction of epidermal H_2O_2 by a narrowband UVB activated pseudocatalase PC-KUS [2] protein expression returns to normal ($-H_2O_2$). This result suggests that calmodulin expression is affected by mM H_2O_2 as shown earlier *in vivo* by FT-Raman spectroscopy [2,3].

In situ immunofluorescence studies. Frozen slides were air dried for 60 min at room temperature before fixing in ice-cold methanol for 6 min and blocked in 10% normal donkey serum (NDS, Jackson ImmunoResearch, Soham, Cambridgeshire, UK) for 90 min following a 5 min wash in PBS. Calmodulin was detected by using a polyclonal rabbit anti-human antibody (Autogen Bioclear, Calne, Wiltshire, UK) diluted 1:50 in 1% NDS followed by incubation at room temperature for 3 h. Then the slides were washed 4× with PBS, air dried and incubated for 1 h with a fluorescent secondary antibody (fluorescein isothiocyanate (FITC) conjugated donkey anti-rabbitat a dilution of 1:100 (Jackson Immunoresearch, Soham, Cambridgeshire, UK.) Slides were washed 3× with PBS air dried and mounted on Vectashield Mounting Medium (Vector, Peterborough, UK). Slides were viewed under a Leica DRMIB/E fluorescence microscope (Wetzlar, Germany) and images were captured using a digital CCD camera (Hamamatsu, Welwyn Garden City, Hertshire, UK) and the IPLab imaging software (Scanalytics, Fairfax, VA, USA).

Molecular structural modelling of calcium binding domains on calmodulin. The structure of calmodulin (ICLL) was obtained from the protein data bank (CAA36839). After oxidation of methionine residues to methionine sulfoxide oxidised calmodulin was modelled using Hyperchem™ software (Hypercube, Gainsville, Florida, USA) followed by Minimisation and Deep View analysis (Swiss Institute for Bioinformatics, Lausanne, Switzerland). The stability of calcium binding sites before and after oxidation was assessed based on the distances for the O-donor ligands in the calcium co-ordination spheres.

^{45}Calcium binding experiments before and after oxidation with H_2O_2. Calmodulin (1.0 mg) was preincubated with $^{45}CaCl_2$ (10^{-3}M) for 30 min to ensure saturation labelling of the protein. The radiolabelled protein was applied to a 1 × 10 cm G-25 Sephadex column and then eluted with distilled water in 1.0 ml fractions. Protein concentration in each fraction was determined at 280 nm by the method of Kalb and Bernlohr [19]. Next, 0.2 ml aliquots were added to 3.0 ml of Ready Safe Scintillation fluid and counted in a Packard Tricarb Scintillation Counter on the ^{14}C channel. The peak radioactive fractions were incubated with 10^{-3} M H_2O_2 for 1 h and the oxidised protein was chromatographed on an identical G25 column and the residual ^{45}calcium was determined using the same procedure as above. Calcium loss was calculated from native protein minus oxidised protein.

Determination of calcium ATPase activity. Membrane-associated calcium ATPase in 3 mm skin biopsies were measured spectrophotometrically using an adaptation of the method used for single muscle fibres [20–22].

The reaction involved coupling the ATP-generating system (i.e., ADP + PEP with pyruvate kinase) to produce pyruvate and steady state ATP) to lactic dehydrogenase by measuring the rate of production of NAD from NADH at 340 nm in the presence of 3 mm skin biopsies held by a miniature sample holder under the surface of the solution in the cuvette. Assays were performed in 1.0 ml containing 2 mM PEP, 2.415×10^{-4} M NADH, 2×10^{-4} M ATP (NB. In this assay we have an ATP generating system so that the optimal steady state concentration of ATP is always present, i.e., 120 U of pyruvate kinase (1 U reacts with 1 μmol of substrate/min), 120 U of lactic dehydrogenase and 1 μg of A23187. The decrease in OD_{340} was measured over 3 min before the addition of 3.5×10^{-6} M $CaCl_2$. The calcium-dependent reaction rate/minute was determined for six patients and six healthy controls.

Results

Decreased epidermal calmodulin expression in patients with vitiligo

In order to test whether calmodulin could be affected by H_2O_2, we first followed the *in situ* protein expression using full skin biopsies from patients with acute vitiligo and after reduction with pseudocatalase PC-KUS for immunofluorescence labelling. The data showed that the expression of calmodulin is significantly decreased in acute patients compared to healthy controls with the same skin phototype (Fitzpatrick classification) [18] (Fig. 1A). After reduction of epidermal H_2O_2, calmodulin expression returned to normal levels (Fig. 1B).

The result suggested that the accumulation of epidermal H_2O_2 in the mM range as shown earlier by *in vivo* FT-Raman spectroscopy, affects the protein expression of calmodulin in acute vitiligo.

This assumption gained further support due to the recovery of protein expression after reduction of epidermal H_2O_2 with pseudocatalase PC-KUS.

H_2O_2 affects ^{45}calcium binding to calmodulin

Next, we decided to follow the stability after H_2O_2-mediated oxidation of calcium binding to calmodulin. For this purpose we utilised ^{45}calcium. The radiolabelled protein was applied to a G-25 Sephadex column (1 × 7 cm) and eluted with distilled water in 1.0 ml fractions. Protein concentrations were determined in each fraction at 280 nm [19] followed by counting on the ^{14}C channel in a Scintillation Counter. The results showed that the protein peak and the ^{45}calcium peak were superimposed. The peak fraction was oxidised with 10^{-3} M H_2O_2 for 1 h at room temperature and then again chromatographed on an identical G-25 Sephadex column. After elution, collection of 1 ml fractions and counting the results showed after oxidation a 93.6% loss of the bound ^{45}calcium (Fig. 2). This result implies a stoichiometric loss of calcium from all four EF-hand binding sites on calmodulin.

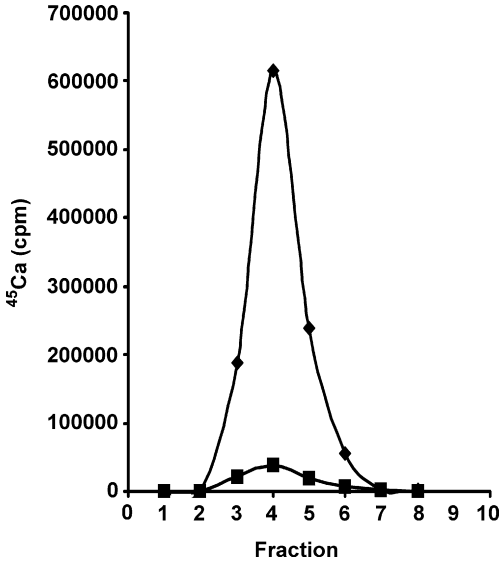

Fig. 2. H_2O_2-mediated oxidation of ^{45}calcium labelled calmodulin yields the loss of all four calcium ions from the four EF-hand binding sites. Oxidation of ^{45}calcium labelled calmodulin by 10^{-3} M H_2O_2 was followed by chromatography on a G-25 Sephadex column showing that calmodulin bound ^{45}calcium eluted in fraction 4. Protein concentrations were determined at 280 nm [19] (♦——♦). The peak fraction was oxidised with 10^{-3} M H_2O_2 for 1 h at room temperature and rechromatographed showing a stoichiometric loss of ^{45}calcium from the protein (■——■).

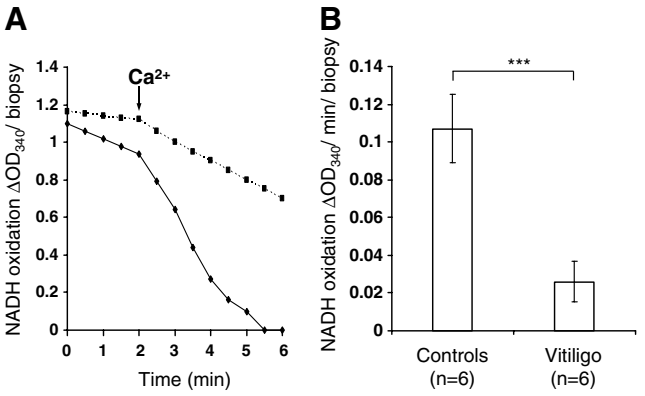

Fig. 3. (A) Comparison of the activities of membrane-associated calmodulin dependent calcium ATPase in 3 mm punch biopsies from active vitiligo skin ($n = 1$) (■——■) and healthy control skin ($n = 1$) (♦——♦). (B) Mean specific activities of calcium-ATPase (±SEM) in patients with vitiligo ($n = 6$) and healthy controls ($n = 6$) ($p < 0.001$).

Epidermal membrane-associated calmodulin-dependent calcium-ATPase activities are low in vitiligo compared to healthy controls

Since it has been shown that after oxidation with H_2O_2 calmodulin fails to activate plasma membrane associated calcium dependent ATPase, we used this enzyme to follow its function in acute and stable vitiligo [15,20], determining its activities in 3 mm punch biopsies obtained from lesional (depigmented) epidermis of patients with acute vitiligo ($n = 6$) and from age matched controls with the same skin phototype III (Fitzpatrick classification) [18] (Fig. 3A and B).These results show a mean 4-fold decrease in activity of this enzyme in the patients compared to healthy controls. In this context it is noteworthy that this enzyme facilitates the uptake of Na^+ and L-phenylalanine, playing a central role in L-phenylalanine turnover to provide sufficient L-tyrosine to support melanogenesis in epidermal melanocytes [16,17].

Structural modelling of calmodulin confirms significant alteration of all four EF-hand binding sites after H_2O_2-mediated oxidation

Based on our results, we simulated the influence of H_2O_2 on the structure of the four classical EF-hand domains in calmodulin [20,23]. Fig. 4A–D presents the comparison of the four calcium binding domains before and after oxidation with H_2O_2. Bond distance measurements to calcium atoms from O-donor residues were assessed and are presented in

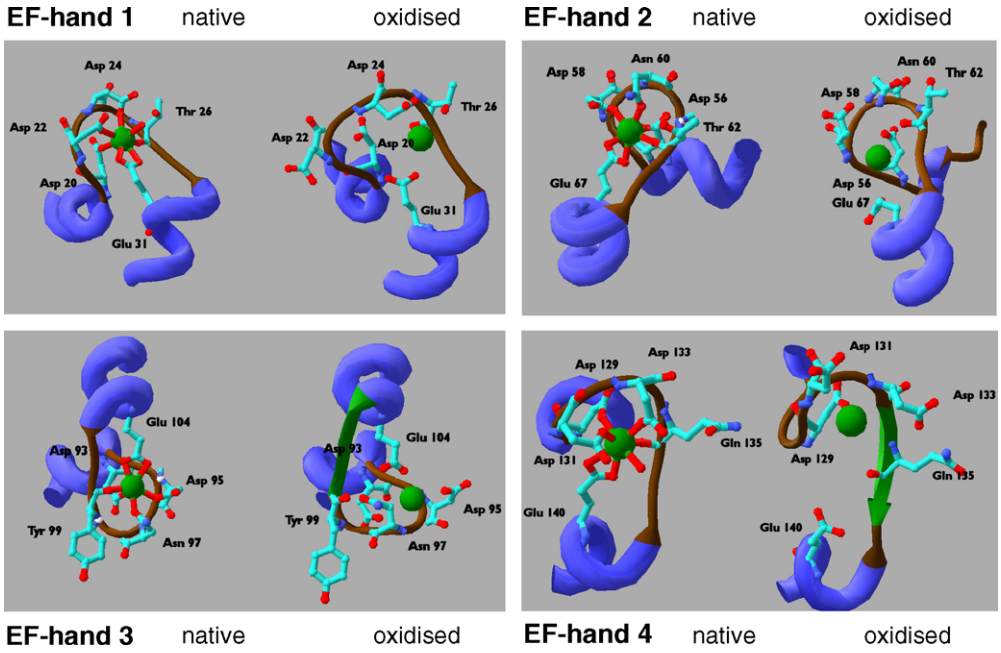

Fig. 4. Computer simulation of H_2O_2-mediated oxidation on calmodulin confirms major changes on all four EF-hand binding sites. The structure of the four EF-hand binding domains on calmodulin determined from the X-ray crystallographic structure before (native) and after its oxidation of Met residues by H_2O_2 to Met-S=O (oxidised; red, O-bonds; green ball, calcium). Bond distances for O-donor atoms to the calcium binding sites before and after oxidation are presented in Table 1. (A) EF-hand 1. Calcium is bound through co-ordination to seven oxygen atoms from the residues Asp^{20}, Asp^{22}, Asp^{24}, Thr^{26}, Glu^{31}, and 1 water molecule. Oxidation of Met residues by H_2O_2 causes conformational changes and shifts in the position of calcium co-ordinating residues leading to complete loss of binding of the Ca^{2+} ion. (B) EF-hand 2. Calcium is bound through co-ordination to seven oxygen atoms from the residues Asp^{56}, Asp^{58}, Gln^{60}, Thr^{62} Glu^{67}, and 1 water molecule. Oxidation of Met residues by H_2O_2 causes conformational changes and shifts in the position of calcium co-ordinating residues leading to complete loss of binding of the Ca^{2+} ion. (C) EF-hand 3. Calcium is bound through co-ordination to seven oxygen atoms from the residues Asp^{93}, Asp^{95}, Asn^{97}, Tyr^{99}, Glu^{104}, and 1 water molecule. Oxidation of Met residues by H_2O_2 causes conformational changes and shifts in the position of calcium co-ordinating residues leading to complete loss of binding of the Ca^{2+} ion. (D) EF-hand 4. Calcium is bound through co-ordination to seven oxygen atoms from the residues Asp^{129}, Asp^{131}, Asp^{133}, Gln^{135}, Glu^{140}, and 1 water molecule. Oxidation of Met residues by H_2O_2 causes conformational changes and shifts in the position of calcium co-ordinating residues leading to complete loss of binding of the Ca^{2+} ion.

Table 1
Bond distances (in Å) for O-donor atoms to the four calcium binding sites for calmodulin before and after oxidation with H_2O_2

AA	Native	Oxidised
EF-hand 1		
Asp^{20}	2.42	6.11
Asp^{22}	2.50	9.55
Asp^{24}	2.39	3.16
Thr^{26}	2.35	2.98
Glu^{31}	2.38	3.15
	2.43	4.11
EF-hand 2		
Asp^{56}	2.13	3.87
Asp^{58}	2.30	5.05
Asn^{60}	2.38	6.48
Thr^{62}	2.29	3.87
Glu^{67}	2.40	4.06
	2.55	5.51
EF-hand 3		
Asp^{93}	2.23	3.57
Asp^{95}	2.53	2.96
Asn^{97}	2.35	4.83
Tyr^{99}	2.20	7.57
Glu^{104}	2.44	3.29
	2.59	5.06
EF-hand 4		
Asp^{129}	2.31	3.01
Asp^{131}	2.45	5.55
Asp^{133}	2.30	5.26
Gln^{135}	2.30	6.18
Glu^{140}	2.55	8.49
	2.57	9.32

Table 1. The result confirmed severe alteration of all four EF-hand calcium binding sites leading to the loss of calcium.

Discussion

Over the last decade vitiligo has emerged as an extremely powerful model disease for H_2O_2-mediated oxidative stress (for review see [3]).

Twenty years ago calcium uptake in keratinocytes established from the lesional skin of patients with vitiligo was shown to be significantly decreased compared to healthy control cells [13]. Later it was recognised that epidermal melanocytes established from the lesional skin of vitiligo patients were also defective in calcium uptake [14]. At that time it was not known whether this defect in calcium homeostasis was caused by its uptake, its release from intracellular stores or its efflux via calmodulin-dependent ATPase. After earlier reports have shown that oxidised calmodulin looses its ability to activate calcium ATPase and since it was later demonstrated that calcium efflux via ATPase was coupled to the active transport of L-phenylalanine, it was tempting to follow the effect of H_2O_2-mediated oxidation on calcium binding of calmodulin to gain further insight into the scenario of acute vitiligo, especially in the light of the documented perturbed calcium homeostasis and as well as the impaired L-phenylalanine uptake in these patients [16,17]. The use of structural computer modelling together with ^{45}calcium binding experiments demonstrated that H_2O_2-mediated oxidative stress disrupts all four EF-hand binding domains on calmodulin. Moreover, our results confirmed in the epidermis of acute vitiligo that oxidised calmodulin leads to a 4-fold decrease of the calmodulin-dependent ATPase activities (Fig. 3).

Taken together these novel data indicate that the continuous accumulation of H_2O_2 in the epidermis of patients with vitiligo disrupts calcium homeostasis in the skin of those individuals, a process which is reversible after correction of the redox balance via a pseudocatalase PC-KUS. Moreover, we here provide a novel mechanism, how oxidised calmodulin looses its functionality due to the loss of the four calcium atoms from its EF-hand binding sites. It is tempting to speculate that the H_2O_2-mediated oxidation of calmodulin with its consequence on the ATPase and other calmodulin dependent processes could be of major importance in other diseases where oxidative stress in general or *in loco* could affect the calcium balance.

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