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Accumulation of nitrotyrosine on the SERCA2a isoform of SR Ca-ATPase of rat skeletal muscle during aging: a peroxynitrite-mediated process?

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Abstract The SR Ca-ATPase in skeletal muscle SR vesicles isolated from young adult (5 months) and aged (28 months) rats was analyzed for nitrotyrosine. Only the SERCA2a isoform contained significant amounts with approximately one and four nitrotyrosine residues per young and old Ca-ATPase, respectively. The in vitro exposure of SR vesicles of young rats to peroxynitrite yielded selective nitration of the SERCA2a Ca-ATPase even in the presence of excess SERCA1a. No nitration was observed during the exposure of SR vesicles to nitric oxide in the presence of O_2. These data suggest the in vivo presence of peroxynitrite in skeletal muscle. The greater nitrotyrosine content of SERCA2a from aged tissue implies an age-associated increase in susceptibility to oxidation by this species.

Key words: Peroxynitrite; Nitric oxide; Nitrotyrosine; Reactive oxygen species; Aging; SR Ca-ATPase

1. Introduction

The post-translational modification of proteins by reactive oxygen species has been recognized as an important feature of biological oxidative stress [1]. Significant levels of such modified proteins can accumulate in aged biological tissue [2–5], eventually paralleled by altered enzyme activities dependent upon the nature of the covalent protein modification. The amount of modified protein present may be used as a biological marker to assess potential levels of oxidative stress as well as the capacity of an organism to cope with such stress. Recently, peroxynitrite ($ONOO^-$) has been identified as a potentially harmful reactive oxygen species due to the high reactivity and selectivity in its reactions with biomolecules such as lipids and proteins [6–8]. Peroxynitrite forms under conditions of simultaneous generation of superoxide and nitric oxide (reaction 1; $k_1 = 4.3\text{–}6.7 \times 10^9\ M^{-1} \cdot s^{-1}$ [9,10]).

$$NO^\bullet + O_2^{\bullet-} \rightarrow ONOO^- \quad (1)$$

Its reaction with tyrosine leads to oxidation, hydroxylation and to *ortho*-nitrotyrosine [11,12]. The latter has been discussed as a biological marker for the assessment of the exposure of tissue to oxidative stress, and, in particular, nitric oxide-derived reactive oxygen species. Significant amounts of nitrotyrosine have, for example, been detected in human atherosclerotic tissue [13].

We have focused on the role of peroxynitrite in the modification of biological tissues during biological aging. The observation that skeletal muscle of aged individuals shows increased relaxation times has been correlated with a potential age-related dysfunction of the sarcoplasmic reticulum (SR) Ca-ATPase [14]. Pending a detailed analytical characterization of possible age-related covalent modifications of this protein, it appears reasonable that reactive oxygen species could be responsible for such modifications since they are actively involved in the modulation of skeletal muscle function [15,16]. For example, superoxide dismutase-sensitive reactive oxygen species have been found to account for 85% of a 6-fold increase of cytochrome *c* reducing equivalents in actively contracting muscle as compared to resting muscle [16], suggesting a specific role for superoxide. Moreover, it has been demonstrated that constitutive nitric oxide synthase is highly expressed in skeletal muscle [17] and that nitric oxide (NO) may modulate skeletal muscle contraction [16].

Due to the simultaneous generation of NO and superoxide we expect that skeletal muscle will be periodically exposed to peroxynitrite. The present paper will demonstrate that during biological aging significant amounts of nitrotyrosine accumulate on the skeletal muscle SR Ca-ATPase and that this modification is selective to the SERCA2a slow-twitch isoform of the Ca-ATPase. In vitro oxidation experiments with skeletal SR vesicles containing both fast- and slow-twitch muscle will show that peroxynitrite is able to selectively nitrate the SERCA2a (slow-twitch) isoform even in the presence of excess SERCA1a (fast-twitch) isoform, and thus we suggest that peroxynitrite may be responsible for the in vivo accumulation of nitrotyrosine on SERCA2a.

2. Materials and methods

2.1. Chemicals

The following chemicals were purchased from the respective sources: monoclonal antibodies to SERCA1 and SERCA2a (Affinity Bioreagents); anti-nitrotyrosine monoclonal antibody (Upstate Biotechnology); goat anti-mouse IgG(H+L) HRP conjugate (Pierce); 3-(*N*-morpholino)propanesulfonic acid (MOPS), soybean trypsin inhibitor, TPCK-treated trypsin, 4-chloro-1-naphthol, 3-nitrotyrosine (Sigma). All other chemicals were analytical grade products. Nitric oxide was prepared in situ by the release of NO from diethylamine/nitric oxide

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Abbreviations: DEA/NO, diethylamine/nitric oxide complex sodium salt; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum.

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complex sodium salt (DEA/NO; Research Biochemicals International), essentially as described by Maragos et al. [18]. Peroxynitrite was synthesized by ozonization of ice-cooled aqueous sodium azide at pH 12, essentially as described [19] (precautions for the procedure are described in ref. [19]). The actual peroxynitrite concentrations were determined before each experiment at λ_{max} = 302 nm taking ε_{302} = 1,670 $M^{-1} \cdot cm^{-1}$ [20].

2.2. Animals and isolation of sarcoplasmic reticulum

Native SR vesicles were prepared from rat hindlimb muscles according to Ferrington et al. [21]. For each preparation, SR membranes were isolated from equal numbers (3 animals per preparation) of young (5 months) and old (28 months) adult male Fisher strain 344 rats obtained from the National Institute of Aging maintained rat colony (Harlan Sprague Dawley, Inc., Indianapolis). Prior to sacrificing, the rats were generally allowed to recover for two weeks after arrival. SR vesicles were suspended in a medium consisting of 0.3 M sucrose and 20 mM MOPS (pH 7.0) and stored at −70°C. Protein concentration was determined by the Lowry method [22] using bovine serum albumin as a standard.

2.3. Polyacrylamide gel electrophoresis

SDS-PAGE was performed using a 5% Laemmli gel with a 3% stacking gel [23], resolving both the fast-twitch and slow-twitch isoforms of Ca-ATPase, SERCA1a and 2a, respectively. Peptide fragments A, A_1, A_2, and B, obtained by limited tryptic digest, were separated on a 10% tricine gel [24]. Gels were either stained for protein with Coomassie blue or electroblotted. The relative amount of Ca-ATPase in the SR preparations was determined from computerized densitometric measurements of the protein band migrating with an apparent molecular weight of 110,000 Da.

2.4. Western blotting of Ca-ATPase isoforms and nitrotyrosine

SR proteins were separated by 5% Laemmli SDS-PAGE or a 10% tricine gel, respectively, and then transferred to nitrocellulose paper (0.4 μm pore size) electrophoretically for 2.5 h at 90 V using 25 mM Tris, 195 mM glycine, and 20% methanol as the transfer buffer. Western blotting was done as previously described [25]. The primary antibodies for SERCA1a, SERCA2a and nitrotyrosine were diluted 1:2000, 1:250, and 1:1000, respectively. The secondary goat anti-mouse IgG-HRP- linked antibody was diluted 1:10,000. Color development of the blots was accomplished using 4-chloro-1-naphthol (3 mg/ml methanol) and 20 μl of 30% H_2O_2 in 20 ml of TBS. Quantitation of the signals was accomplished by computerized densitometry (area × intensity). Amounts of 50–200 μg of SR protein provided a linear response for the quantitation of the SERCA2a antibody.

2.5. Preparation of protein for amino acid analysis

The SERCA2a was purified by preparative SDS gel electrophoresis (5% separating gels, 165 × 165 × 0.8 mm according to ref. [26]). After electrophoresis the gels were stained for 30 min with freshly prepared 0.1% Coomassie Blue R-250 in 10% methanol and 0.5% acetic acid and subsequently destained with 10% methanol (4 × 5 min). The band corresponding to SERCA2a (as identified by labeling with SERCA2a antibody; see below) was cut out and washed 10 min in the electroelution buffer (20 mM Tris, pH 8.3, 150 mM glycine, 0.1 mM DTT, 0.025% SDS). The samples were frozen overnight before elution in a Little Blue Tank electroelutor (ISCO). Before collection of the samples, the current was reversed for 30 s to desorb the proteins from the membranes. Salts and SDS were removed as described [26].

2.6. Amino acid analysis

Amino acid analysis was done after hydrolysis of the protein in (i) 6 N HCl, and (ii) 4 N methanesulfonic acid, respectively (20 h, 110°C). Services were provided by Commonwealth Biotechnologies, Inc. (Richmond, VA).

2.7. Limited tryptic digestion

SR vesicles (4 mg/ml SR protein) were digested with trypsin at a ratio of trypsin/SR protein of 1:100 up to 35 min at 25°C in 80 mM KCl, and 20 mM MOPS, pH 7.0 [27]. The reaction was stopped by addition of soybean trypsin inhibitor (type 1-S, Sigma) at an inhibitor/trypsin ratio of 2:1.

2.8. HPLC of nitrotyrosine

For the analysis of nitrotyrosine, an aliquot of SR protein was hydrolyzed for 20 h at 110°C in a gas phase hydrolyzer before HPLC analysis was performed according to the method described in reference [28]. In brief, samples were separated on an Inertsil ODS-3 column (GL Sciences, Inc.), eluted isocratically at 0.7 ml/min with a mixture of 0.03 M sodium acetate and 0.03 M sodium citrate, pH 3.6. Nitrotyrosine eluted with t_R = 54.0 ± 0.3 min, as monitored by parallel UV-detection at 275 and 350 nm. We observed a linear response and satisfactory signal to noise level of nitrotyrosine standards at concentrations of [NO_2-Tyr] ≥ 0.1 μM. Furthermore, in control experiments hydrolysates of SR protein were spiked with authentic *ortho*-nitrotyrosine.

2.9. Statistics

For the in vivo determinations of nitrotyrosine 5 groups of animals, each of them containing 3 rats, were sacrificed for young and old rats, respectively. The values in the Figures and the Table are expressed as mean ± S.E.M for n experiments. Groups were compared by t-test. Significance was set at $P < 0.01$.

3. Results

3.1. In vivo formation of nitrotyrosine during aging

Fig. 1A demonstrates that SR vesicles, obtained from aged (28 months old) rats contain nitrotyrosine essentially only on the SERCA2a isoform of the SR Ca-ATPase and not on the SERCA1a isoform, as indicated by the immunoreactivity of the corresponding protein bands with both the nitrotyrosine and SERCA2a antibodies. Similar results were obtained for

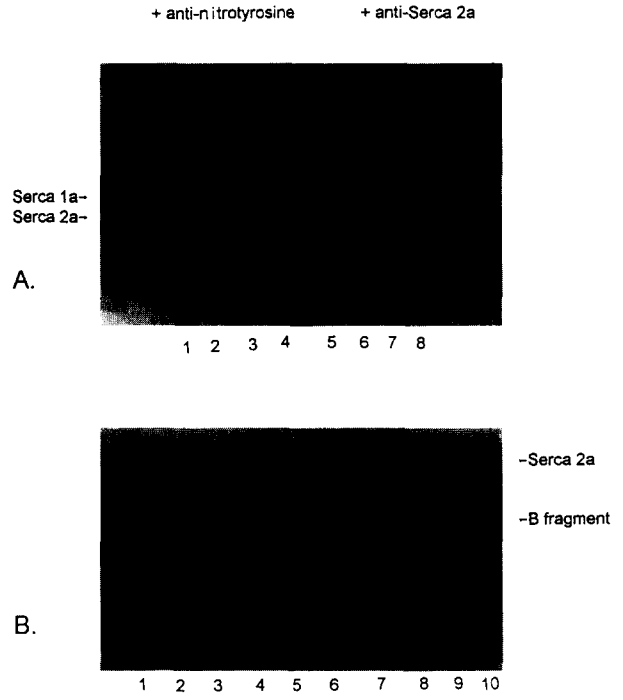

Fig. 1. (A) Immunodetection of nitrotyrosine and SERCA2a in SR vesicles from aged rats (28 months old). Lanes 1 and 6 (prepared in winter), lanes 2 and 7 (prepared in spring), lanes 3 and 8 (prepared in summer). The amount of applied protein was 100 μg per lane. Lanes 4 and 5: molecular weight standards (Bio-Rad): 205 kDa, 121 kDa, 86 kDa and 50.7 kDa. (B) Immunodetection of nitrotyrosine in the major tryptic fragments of SERCA2a. Lane 1: Molecular weight standard (Bio-Rad): 205, 121, 86, 50.7, 33.6, 27.8, 19.4 and 7.4 kDa. Lane 2: trypsin (5 μg) + trypsin inhibitor (10 μg) (control experiment). Lanes 3–10: Aliquots of SR vesicles after different times of digestion: 0 min (lane 3), 5 min (lane 4), 10 min (lane 5), 15 min (lane 6), 20 min (lane 7), 25 min (lane 8), 30 min (lane 9), 35 min (lane 10).

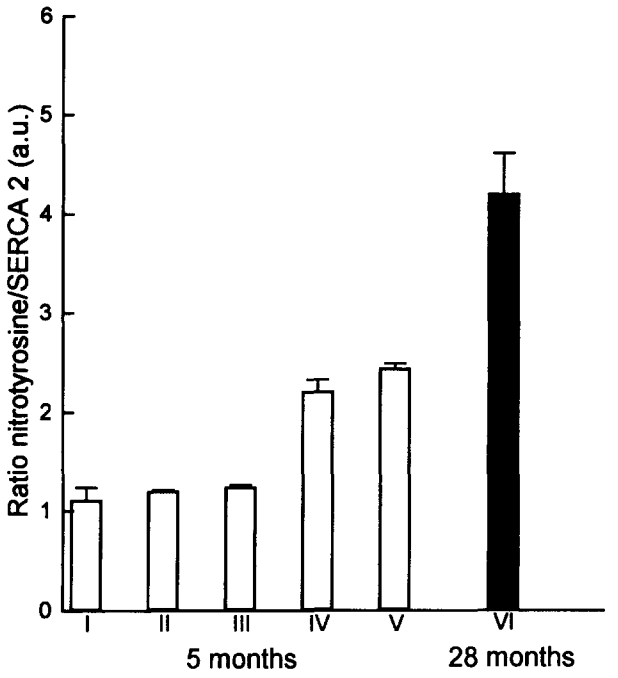

Fig. 2. Ratio of nitrotyrosine/Serca2a in SR vesicles of young (5 months old; panels I–V) and old (28 months old; panel VI) rats, determined by immunoreactivity. Young rats: control, (I); exposed to NO/O_2, (II); exposed to 56 μM peroxynitrite, (III); exposed to 200 μM peroxynitrite, (IV); exposed to 200 mM peroxynitrite in the presence of 2 μM SOD, (V). Panels are significantly different at the following levels: I and II, $P < 0.5$; I and III, $P < 0.01$; I and IV, $P < 0.001$; IV and VI, $P < 0.05$.

SERCA2a of young (5 months old) rats (blot not shown). The densitometric analysis of the Western blots shown in Table 1 (second column) reveals that SR vesicles from aged rats display a nitrotyrosine/SERCA2a ratio which is 4-fold higher in old as compared to young rats. Thus, SERCA2a Ca-ATPase of aged rats is characterized by elevated levels of nitrotyrosine.

Our SR preparations contain approximately 40% Ca-ATPase relative to total SR protein, with approximately 10% of the Ca-ATPase content being the SERCA2a isoform, as estimated from the area of the Ca-ATPase band reacting with the SERCA2a antibody (Ferrington et al., unpublished data). Importantly, SR preparations were nearly identical; aged rats had slightly less (4%) total Ca-ATPase protein compared to young rats (Ferrington et al., unpublished data). With a molecular weight of SERCA2a of 109,477 Da [29] we calculate that 1 mg of SR protein contains 0.37 nmol of SERCA2a. When SR vesicles were subjected to gas phase hydrolysis followed by HPLC analysis, a content of 0.52 ± 12 nmol nitrotyrosine/mg SR protein was obtained for young rats (see Table 1), corresponding to a molar ratio for nitrotyrosine/SERCA2a of 1.4 ± 0.33. This is close to the ratio of 1.11 determined by Western blot analysis (see Table 1). For aged rats we obtained an absolute content of 1.43 ± 0.16 nmol nitrotyrosine per 1 mg SR protein, corresponding to a molar ratio for nitrotyrosine/SERCA2a of 3.9 ± 0.4. This value matches the ratio obtained by the densitometry of the Western blots. Amino acid analysis of purified SERCA2a reveals a loss of two tyrosine residues from SERCA2a isolated from old rats (see Table 1). The number of 18 ± 0.7 tyrosine residues for young rats corresponds to the published sequence of SERCA2a [29]. We suspect that the amount of 1.4 nitrotyrosine residues, measured by HPLC for SERCA2a from young rats, may partially be accounted for by the error limit of ±0.7 of the amino acid analysis.

In summary, three independent analysis reveal that skeletal muscle SR vesicles, isolated from aged rats, are characterized by a 2- to 4-fold higher relative amount of nitrotyrosine as compared to young rats, corresponding to approximately 2–4 nitrotyrosine residues per SERCA2a monomer.

3.2. The localization of nitrotyrosine

The limited proteolysis of SR protein (containing both SERCA1a and SERCA2a) yields subfragments A, A_1, A_2, and B [27]. Fig. 1B displays the results of immunoblotting of these respective fragments from aged rats with the nitrotyrosine antibody. It is apparent that the nitrotyrosine residues are located predominantly on the B-fragment which comprises residues Thr^{506}–Glu^{997} and contains 9 out of the total 18 tyrosine residues [29]. We note a slight immunoreactivity of the A_1-fragment whereas no immunoreactivity is observed for the A-fragment (comprising A_1+A_2). At present we cannot conclusively explain this observation except assuming that a large excess of non-nitrotyrosine-containing A-fragment from SERCA1a may mask small amounts of nitrotyrosine-containing A-fragment of SERCA2a on the blot. There is significant difference in charge of the smaller A_1-fragments from SERCA1a (−3) and SERCA2a (+3) which may be sufficient for partly resolution on the blot, eventually permitting immunoreactivity of the SERCA2a A_1-fragment with the nitrotyrosine antibody.

3.3. The reaction of skeletal muscle SR vesicles with peroxynitrite

We reacted SR vesicles (10 mg SR protein/ml) isolated from young rats, with (i) 56 μM peroxynitrite, (ii) 200 μM peroxynitrite, and (iii) 200 μM peroxynitrite in the presence of 2 μM Cu, Zn superoxide dismutase (10 mM potassium phosphate, treated with Chelex-100, pH 7.3, 100 mM NaCl, 25°C). The addition of minute amounts of alkaline peroxynitrite stock solutions shifted the pH of the reaction mixtures by less than 0.2 pH units. Fig. 2 displays the respective yields of nitrotyrosine, expressed as ratio of nitrotyrosine/SERCA2a, obtained by densitometric analysis of Western blots, essentially as described for Fig. 1. Panel I displays the results of a control experiment in which a peroxynitrite solution was allowed to decompose [30] before it was combined with SR vesicles ('reverse-order-of-addition experiment' [30]). The amount of detected nitrotyrosine was identical to that measured for young SR vesicles in the absence of peroxynitrite, indicating that peroxynitrate decomposition products did not contribute to the formation of

Table 1
Nitrotyrosine content in rat skeletal SR preparations

Age	Nitrotyrosine/Serca2a ration[a]	Nitrotyrosine, nmol/mg SR protein[b]	Tyrosine, res./mol Serca 2a[c]
5 months	1.11 ± 0.13	0.52 ± 0.12	18 ± 0.7
28 months	4.20 ± 0.41	1.43 ± 0.16	16 ± 0.9

[a] Data are obtained by densitometry of Western blot.
[b] Data obtained by reversed-phase HPLC.
[c] Data obtained by amino acid analysis. Samples of 5 and 28 month old rats are significantly different at the level of $P < 0.01$.

nitrotyrosine. The exposure of 10 mg SR protein/ml to 56 μM peroxynitrite (panel III) shows little elevation of nitrotyrosine levels over that of the control experiment. However, 200 μM peroxynitrite (panel IV) form significant yields of nitrotyrosine which can be slightly enhanced by the addition of SOD (panel V). It has been shown that SOD can catalyze the nitration of tyrosine [11]. However, the small effect of SOD on the nitration of SERCA2a may be rationalized by the large size of the membrane protein potentially hampering interaction of a peroxynitrite-SOD complex with tyrosine residues of SERCA2a.

There was no significant formation of nitrotyrosine when air-saturated mixtures containing 10 mg SR protein/ml were exposed over 20 min to 200 μM DEA/NO at 25°C (panel II). DEA/NO yields 1.5 equivalents of NO per DEA/NO [18].

4. Discussion

Significant levels of nitrotyrosine accumulate on the SERCA2a isoform of Ca-ATPase during biological aging. However, also SERCA2a isolated from young rats carries an average of 1.4 nitrotyrosine residues per protein monomer. The exposure of SR vesicles to peroxynitrite results in the selective nitration of tyrosine residues of the SERCA2a isoform even in the presence of an excess content of SERCA1a. These observations suggest that the isolation of selectively nitrated SERCA2a cannot be rationalized by a potentially fast protein turnover of nitrated SERCA1a (escaping detection) but may represent structural differences of the two isoforms rendering the SERCA2a tyrosine residues more susceptible to the reaction with peroxynitrite (we note that SERCA2a carries only 18 tyrosine residues [29] as compared to 23 tyrosine residues of SERCA1a [31]). These results are corroborated by recent more mechanistical studies in which we had shown that the in vitro reaction of peroxynitrite with rabbit SERCA1a in SR vesicles did not result in the nitration of tyrosine residues but rather the oxidation of protein thiols [25].

The fact that nitrotyrosine was identified in SERCA2a of both young and old rats suggests that significant levels of peroxynitrite are formed under physiological conditions of muscle contraction and relaxation through simultaneous generation of modulating levels of superoxide and NO [16]. Higher yields of nitrated SERCA2a in aged tissue may be explained by a generally less effective antioxidant defense system of aged organisms [32]. Thus, nitrated SERCA2a may serve as a biological marker for both the exposure of skeletal muscle to peroxynitrite as well as for its capability to buffer (scavenge) reactive oxygen species such as peroxynitrite.

Aerobic (air-saturated) mixtures containing NO at total concentrations similar to the employed peroxynitrite concentrations did not promote nitrotyrosine formation. This may be explained by the competitive reaction of other amino acid residues with NO-derived reactive intermediates different from peroxynitrite and/or before peroxynitrite formation may occur. Wink et al. have investigated NO/O_2 systems, and their kinetic measurements postulate a yet unidentified intermediate which reacts approximately 150 times faster with sulfhydryl groups than with a standard peptide alanyltyrosine [33]. A rate constant of $k_2 > 3.4 \times 10^3\ M^{-1} \cdot s^{-1}$ has been estimated for the forward reaction of equilibrium 2 [34], the primary equilibrium encountered by NO at $[O_2] \gg [NO]_{steady\text{-}state}$ ($k_{NO + NO} > 2 \times 10^3\ M^{-1} \cdot s^{-1}$ [34]).

$$NO + O_2 \rightleftharpoons ONOO^{\bullet} \quad (2)$$

The resulting peroxylradical, $ONOO^{\bullet}$, is expected to oxidize thiols but most probably via an overall two-electron oxidation (reaction 3) [35]. The product $^{\bullet}NO_2$ reacts efficiently with thiols [36] with an estimated overall rate constant in aqueous solution of $k_4 > 5 \times 10^8\ M^{-1}s \cdot^{-1}$ [37], where reaction 4 proceeds via intermediary adducts such $CysS^{\bullet}(NO_2)H$ and *S*-nitroso thiols [36]. ($CysS^-$ = cysteine thiolate.)

$$ONOO^{\bullet} + RSH \rightarrow {}^{\bullet}NO_2 + RSOH \quad (3)$$

$$^{\bullet}NO_2 + CysS^- \rightarrow\rightarrow NO_2^- + CysS^{\bullet} \quad (4)$$

Thus, protein thiols may efficiently compete for NO-derived reactive species in the NO/O_2 system before any nitration can occur, and experiments are currently underway to test this hypothesis. Future experiments will now focus on the physiological significance of the nitration of the SERCA2a isoform of skeletal muscle Ca-ATPase by peroxynitrite.

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