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TITOLO DELLA RELAZIONE: Role of oxidative stress in TRPA1-dependent painful states induced by third-generation aromatase inhibitors.

RELAZIONE:

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Relazione finale sull'attività svolta per la realizzazione del Progetto di Ricerca:

"**Role of oxidative stress in TRPA1-dependent painful states induced by third-generation aromatase inhibitors**".

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1 – Introduction

The third-generation aromatase inhibitors (AIs) exemestane, letrozole and anastrozole have proven to be very active drugs in the treatment of hormone receptor-positive breast cancer [1, 2]. AIs act by blocking the activity of the almost ubiquitous enzyme aromatase, which is responsible for the aromatization of androgens into estrogens, which target cancer cells promoting their replication and growth [3]. Despite AIs are generally well tolerated with limited serious adverse events, a number of side effects that can lead to nonadherence has been reported [4]. Among these, AIs-associated musculoskeletal symptoms (AIMSS) are the most frequent [5, 6]. In addition to musculoskeletal pain, pain symptoms associated with AIs have recently been more accurately described with the inclusion of neuropathic, diffused and mixed pain [7].

The Transient Receptor Potential Ankyrin 1 (TRPA1) channel is a polymodal sensor activated by chemical, mechanical and thermal stimuli, expressed by a subpopulation of nociceptive neurons [8]. TRPA1 is activated by pungent molecules and by a series of reactive molecules produced at sites of inflammation and tissue injury, including reactive oxygen (ROS), nitrative or carbonyl species, which activate the channel reacting with key cysteine residues [9, 10]. We recently showed that exemestane (with highly electrophilic conjugated Michael acceptor groups), letrozole and anastrozole (with nitrile moieties), interacting with cysteine residues, activate TRPA1 and cause pain, including AIMSS [11].

A large proportion, up to 40%, but not all patients exposed to AIs develops AIMSS. Therefore, only a fraction of the exposed population exhibits AIMSS, thus implying that additional co-factors should contribute the painful adverse reaction. These co-factors may include classical proinflammatory mediators [12] and also oxidative stress byproducts that are known to be generated by AIs and to target TRPA1. Thus, we hypothesized that the administration of third-generation AIs may be also related to the development of oxidative stress byproducts that lead to the onset of painful states through the activation of TRPA1 channel. Additional co-factors may be of hormonal origin. As treated patients are mainly female and are under stressful conditions, we investigated whether sex hormones and stress-related hormones could target TRPA1.

2 – Results

Systemic exemestane and letrozole induce painful state through the generation of oxidative stress.

We recently showed that systemic injection of the steroidal third-generation AI exemestane (5 mg/kg) in C57BL/6 mice promoted the onset of a mechanical hypersensitivity, which is maximum 3 hours after injection, and disappears 48 hours after the injection [11]. This effect was transiently reverted by systemic pretreatment with the selective TRPA1 antagonist, HC-030031 [11]. Here we showed that the systemic pretreatment with an anti-oxidant agent, α-lipoic acid (100 mg/kg, i.g.), was also able to transiently revert this effect (Figure 1A), suggesting that oxidative stress is involved in this phenomenon. Similar results have been obtained with systemic administration of letrozole, a non-steroidal third-generation AIs. In fact, the treatment with letrozole (0.5 mg/kg, i.p.) evoked a prolonged mechanical hypersensitivity, in C57BL/6 mice, peaking at 3-6 hours and ending completely 48 hours after [10]. This effect was transiently reverted by α-lipoic acid pretreatment (100 mg/kg, i.g.) (Figure 1B).

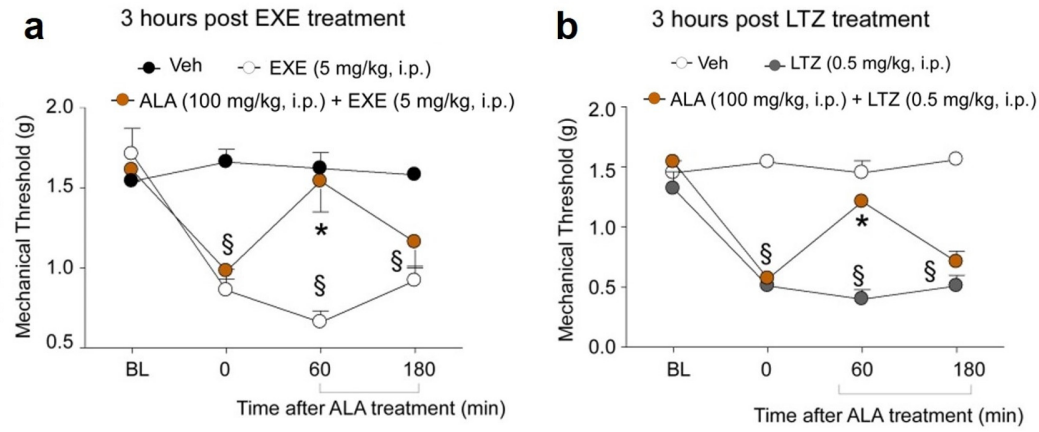

Figure 1. Sistemic exemestane (EXE) and letrozole (LTZ) induce mechanical allodynia *via* oxidative stress generation. Systemic EXE (5 mg/kg, i.p.) (a) and LTZ (0.5 mg/kg, i.p.) (b) induce the onset of mechanical allodynia in C57/BL6 mice, measured using Von Frey hair test, that is transiently abated by pretreatment with α-lipoic acid (ALA, 100 mg/kg, i.g.). Data are mean ± SEM of at least 5 mice for each group. § $P < 0.05$ vs. Veh, * $P < 0.05$ vs. EXE and LTZ Student's T test. BL, basal level.

To verify whether AIs were able to induce the generation of oxidative stress, we measured the levels of hydrogen peroxide (H_2O_2) in the sciatic nerve of C57BL/6 mice after the intraperitoneal administration of letrozole (0.1 - 0.5 mg/kg). Results showed that letrozole (0.5 mg/kg, i.p.), 3 hours after treatment, produced an increase of H_2O_2 levels (Figure 2).

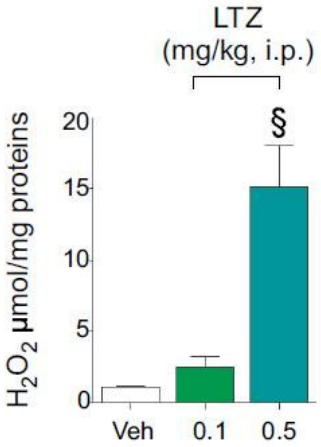

Figure 2. Hydrogen peroxide (H_2O_2) levels are increased in C57BL/6 mice sciatic nerve homogenates after systemic administration of letrozole (LTZ). Intraperitoneal injection of letrozole (0.1-0.5 mg/kg) induces an increase of H_2O_2 levels in C57BL/6 mice sciatic nerve. H_2O_2 levels were detected by using the phenol red-HRPO method [20]. Results represent mean ± s.e.m. of at least 5 mice for each group. Veh is the vehicle of LTZ. §P<0.05 *vs.* Veh; ANOVA followed by Bonferroni *post hoc* test.

Next, to investigate whether letrozole-induced H_2O_2 generation could contribute to the onset of painful states, C57BL/6 mice received intraplantar (i.pl.) injection of H_2O_2 (0.05, 0.5 and 2 µmol/20 µl per paw). H_2O_2 evoked a dose-dependent delayed mechanical allodynia that starts 30 minutes after and lasts for 12 hours after the injection (Figure 3a). The effect evoked by H_2O_2 was almost completely prevented by intraperitoneal (i.p.) pretreatment with the selective TRPA1 antagonist, HC-030031 (100 mg/kg), but not with the selective TRPV1 antagonist, capsazepine (4 mg/kg) (Figure 3b). Thus, by using pharmacological tools, we demonstrated that local administration of H_2O_2 produces a typical TRPA1-dependent behaviour, characterized by a delayed mechanical allodynia.

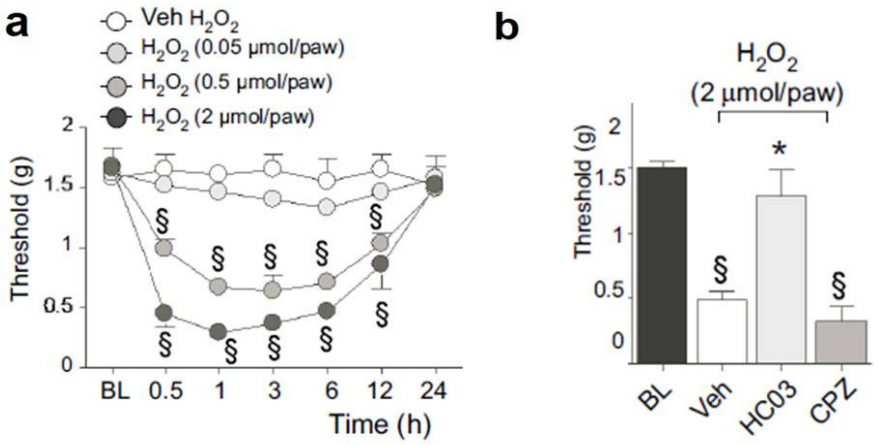

Figure 3. Local administration of hydrogen peroxide (H_2O_2) induces mechanical allodynia *via* TRPA1 activation. (**a**) Intraplantar (i.pl., 20 µl) injection of H_2O_2 (0.05-2 µmol) in C57BL/6 mice produces a dose-dependent mechanical allodynia that starts 30 minutes and lasts 12 hours after the i.pl. injection. (**b**) The mechanical allodynia induced by H_2O_2 (60 minutes after i.pl. injection) is prevented by systemic HC-030031 (HC03, 100 mg/kg, i.p.), but not by capsazepine (CPZ, 4 mg/kg, i.p.). Results represent mean ± s.e.m. of at least 5 mice for each group. Veh is the vehicle of H_2O_2. §P<0.05 *vs.* Veh or BL values, *P<0.05 *vs.* H_2O_2; ANOVA followed by Bonferroni *post hoc* test.

These data were supported by electrophysiology experiments that recapitulate previous findings showing that H_2O_2 may directly target TRPA1 channel [16]. Here we verified whether H_2O_2 could stimulate nociceptive sensory neurons *via* TRPA1 activation, using primary culture of rat dorsal root ganglion (DRG) neurons. H_2O_2 (50-1000 µM) produced a concentration-dependent inward current response in a proportion of cells that responded to the selective TRPV1 agonist, capsaicin (0.1 µM) (Figure 4). The responses evoked by the H_2O_2 (500 µM) were abated by HC-030031 (50 µM) (Figure 4).

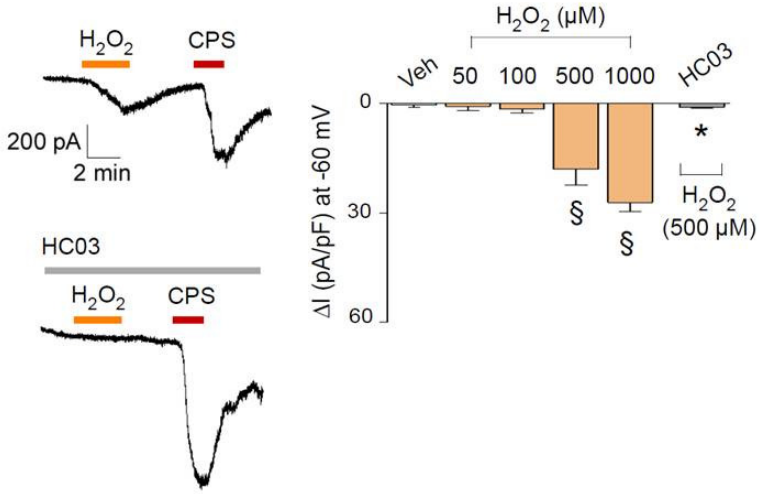

Figure 4. Hydrogen peroxide (H_2O_2) selectively activates the native TRPA1 channel expressed in rat dorsal root ganglion (DRG) neurons. Typical traces and pooled data obtained by whole-cell patch-clamp recordings in rat DRG neurons. Exposure to H_2O_2 (50-1000 μM) elicits a concentration-dependent inward current at -60 mV in capsaicin-sensitive neurons. The selective TRPA1 antagonist, HC-030031 (HC03; 50 μM), abolishes currents evoked by H_2O_2 (500 μM), but not by capsaicin (CPS, 0.1 μM). Results are mean ± s.e.m. of at least 5 cells tested for each experimental condition. §P <0.05 vs. Veh, **P*<0.05 *vs*. H_2O_2 500 μM; ANOVA and Bonferroni *post hoc* test.

In order to assess whether both AIs administration and AIs-dependent oxidative stress production could cooperate to the onset of painful states, we explored the ability of exemestane and letrozole to increase mechanical allodynia to H_2O_2. We found that 10 min after H_2O_2 injection (0.5 μmol/20 μl per paw) administration of exemestane (5 mg/kg, i.p) and letrozole (0.5 mg/kg, i.p) evoked, 3 hours after treatment, a mechanical allodynia markedly increased as compared with exemestane and letrozole-treated mice (Figure 5a,b). The exaggerated responses to AIs were inhibited by α-lipoic acid (100 mg/kg, i.g.) (Figure 5a,b). Thus, the oxidative stress produced by AIs administration results in the potentiation of the AIs-evoked proalgesic mechanism.

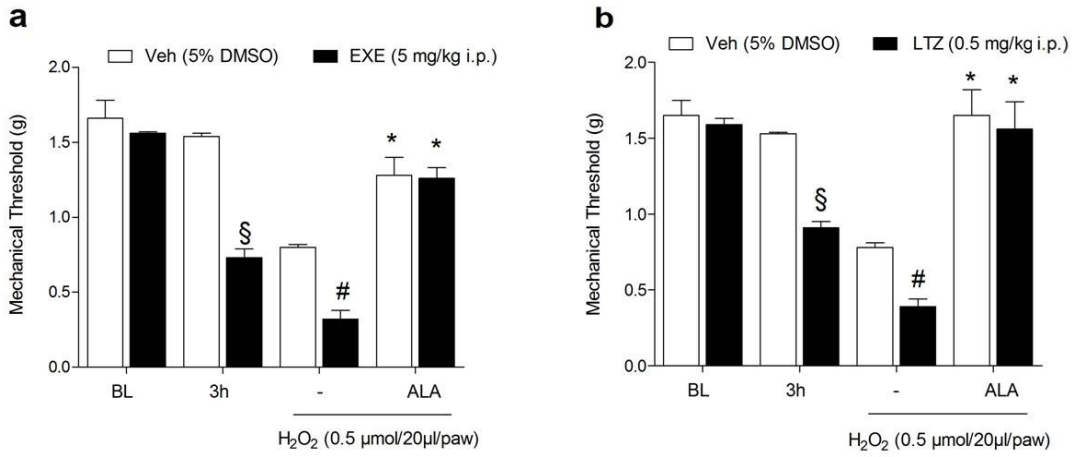

Figure 5. Mechanical allodynia induced by systemic administration of exemestane (EXE) or letrozole (LTZ) is enhanced by hydrogen peroxide (H_2O_2). Intraplantar (i.pl.; 20 µl) injection of H_2O_2 enhances mechanical allodynia produced by EXE (5 mg/kg, i.p.) or LTZ (0.5 mg/kg, i.p.) 3 hours after their administration. The potentiated responses to EXE or LTZ are markedly attenuated by α-lipoic acid (ALA, 100 mg/kg, i.g.). Results represent mean ± s.e.m. of at least 5 mice for each group. Veh is the vehicle of EXE or LTZ, $^{\S}P<0.05$ *vs*. BL values, $^{\#}P<0.05$ *vs*. EXE or LTZ, $^{*}P<0.05$ *vs.* EXE/H_2O_2 or LTZ/H_2O_2; ANOVA followed by Bonferroni *post hoc* test.

Sex and stress-related hormones do not target TRPA1 channel.

In *in vitro* electrophysiological experiments, we found that testosterone (TST, 100 µM), aldosterone (ALD, 100 µM), 17α-hydroxyprogesterone (17αHP, 100 µM), progesterone (PRG, 100 µM), dehydroepiandrosterone (DHEA, 100 µM), cortisol (CRT, 100 µM), and deoxycortisol (DOCRT, 100 µM) did not produce inward currents in HEK293 stably transfected with the human TRPA1 cDNA (Figure 6a,b).

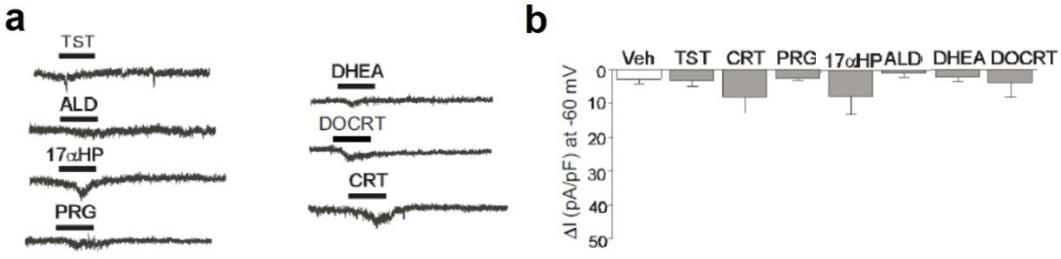

Figure 6. Sex and stress-related hormones do not activate TRPA1 channel. Typical traces (a) and pooled data (b) showing that testosterone (TST, 100 µM), aldosterone (ALD, 100 µM), 17α-hydroxyprogesterone (17αHP, 100 µM), progesterone (PRG, 100 µM), dehydroepiandrosterone (DHEA, 100 µM), cortisol (CRT, 100 µM), and deoxycortisol (DOCRT, 100 µM) did not evoke any inward current at -60 mV in HEK293 stably transfected with human TRPA1 channel. Values are mean ± SEM of at least five cells for each experimental condition.

3 – Discussion

Our previous work shows that TRPA1 mediates AIs-induced painful conditions [11]. Indeed, systemic pretreatment with the selective TRPA1 antagonist transiently reverted mechanical hypersensitivity evoked by these anticancer drugs. Here we reported that the effects of exemestane and letrozole systemic administration were also transiently reverted by the pretreatment with an anti-oxidant agent, α-lipoic acid, suggesting that the generation of oxidative stress byproducts following exemestane and letrozole treatment represents another possible mechanism for the induction of painful states. Our results are also supported by other findings showing that exemestane and letrozole are capable of producing ROS. Despite other controversies [13], a recent investigation has reported that exemestane produces a significant increase in caspase-9 activity and production of intracellular ROS in a breast cancer cell line [14]. These findings suggest that, in addition to the blockage of estrogen biosynthesis, ROS generated by this drug may also participate in inducing an irreversible growth arrest of neoplastic cells. Similarly to exemestane, it has been shown that letrozole, in a murine polycystic ovary model, induces cellular lipid peroxidation and peroxynitrite generation [15].

It is well established that TRPA1 serves as a major sensor for oxidant species. Indeed, diverse series of oxidative stress byproducts has been reported to gate TRPA1, and through this mechanism produce nociceptive responses. These reactive agents include H_2O_2 [16], hypochlorite [17], nitroleic acid [18], acrolein [19] and 4-hydroxynonenal [10] which are lipid peroxidation byproducts. Thus, we propose that exemestane and letrozole contribute to the onset of painful states not only by directly gating TRPA1 channel expressed in sensory neurons, as we previously reported [11], but also through the generation of oxidative stress byproducts.

In conclusion, we propose that third-generation AIs treatment results in the generation of oxidative stress. Thus, AIs (by their direct ability to gate the TRPA1) and reactive species generated by AIs (also capable of stimulating the channel) may cooperate to activate/sensitize TRPA1 expressed by nociceptors. As antioxidants present remarkable pharmacokinetics bias, it should be of great interest to develop therapies that combine the use of the aromatase inhibitors with TRPA1 antagonists so to revert the neurotoxic effects without affecting the anticancer properties of the drugs.

Aromatase inhibition, while reducing downstream production of estrogens, moderately increases upstream plasma concentrations of androgens. Here we examined the ability of different sex and stress-related hormones to directly gate the TRPA1 channel. Data showed that none of the studied compounds was able to target the channel. However, the identification of other steroid hormones upstream to aromatase, whose plasma levels could be altered after AIs

administration, will represent our future perspective to better define the underlying mechanisms involved in AIs-induced painful states.

4 - References

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