from day 0 slightly increased the percentage of beating EBs at days 5 and 8.

**Conclusions:** The time-dependent expression of NOS/sGC/cGKI during cardiac differentiation suggests a potential role of these pathways to trigger cardiomyogenesis. Further functional data will clarify its role in cardiac maturation.

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## Effect of suberoylanilide hydroxamic acid (SAHA) on functional properties of HL-1 cardiomyocytes

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**Objectives:** Histone deacetylases (HDACs) and acetyltransferases (HATs) are enzymes that act removing or adding, respectively, acetyl groups from lysines of histones and a number of other regulatory and structural proteins. Deregulated HAT and HDAC activity plays a role in the development of a wide range of cancers and modulators of these enzymes have been developed as anticancer agents. Suberoylanilide hydroxamic acid (SAHA, commercial name: Vorinostat), a general HDAC inhibitor, was approved for the treatment of refractory cutaneous T-cell lymphoma. Administration of Vorinostat to cancer patients has been associated to QT prolongation and torsade de point development. However, other studies report no cardiac adverse effects of Vorinostat administration on oncological patients. The aim of the study was to evaluate the acute effect of SAHA on functional properties of HL-1 cardiomyocytes, in order to assess its cardiac safety.

**Materials and methods:** HL-1 cells were perfused with 2.5 μM SAHA and the electrophysiological properties, namely action potentials (APs) and sodium calcium exchanger (NCX) current, were investigated by whole-cell configuration of patch-clamp technique. To assess the effect of SAHA on intracellular calcium dynamics, HL-1 was loaded with the Fluo-4 AM calcium indicator and fluorescence detected with an imaging system.

**Results:** The acute administration of SAHA to HL-1 cells caused the appearance of early after depolarizations (EADs) and delayed after depolarizations (DADs) in spontaneous APs and the reduction of NCX current, recorded as nichel sensitive current, from −70 to −40 mV ($p < 0{,}01$ $n = 25$). SAHA affected intracellular calcium dynamics causing a transient increase of intracellular calcium, characterized by a significantly higher amplitude compared to spontaneous calcium transients typical of HL-1 cells, and the subsequent block of spontaneous calcium transients. The effect on calcium transient was prevented in the absence of extracellular calcium. Nichel, a nonspecific inhibitor of NCX and T-type calcium channel (CaT), but not ML218, a specific inhibitor of CaT, mimicked the effect of SAHA on cytosolic calcium dynamics.

**Conclusions:** Herein we provide evidence that acute SAHA administration in HL-1 cells affects NCX function and its regulation plays a role in the after-depolarization appearance and calcium dynamic alterations.

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## Acetylcholine induces nitric oxide production by inducing intracellular $Ca^{2+}$ oscillations in mouse brain endothelial cells

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**Objectives:** Basal forebrain neurons control intracortical arterioles by releasing acetylcholine (Ach), which stimulates endothelial cells (ECs) to produce the vasodilating gasotransmitter, nitric oxide (NO). Surprisingly, the mechanism by which Ach induces NO synthesis in brain ECs is still unknown. An increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) recruits a multitude of endothelial $Ca^{2+}$-dependent pathways, such as $Ca^{2+}$/calmodulin endothelial NO synthase (eNOS). The present investigation sought to investigate the role of intracellular $Ca^{2+}$ signaling in Ach-induced NO production in bEnd5 cells, an established model of mouse brain microvascular ECs.

**Materials and methods:** Changes in $[Ca^{2+}]_i$ were monitored from bEnd5 cells loaded with the $Ca^{2+}$-sensitive dye, Fura-2/AM (2 μM, 30 min), by using a CCD camera. NO was measured by loading the cells with the NO-sensitive fluorochrome, DAF-FM diacetate (10 μM, 1 h).

**Results:** Ach induced dose-dependent asynchronous $Ca^{2+}$ oscillations in bEnd5 cells, 300 μM being the most effective dose to generate a prolonged (up to 1 h) $Ca^{2+}$ burst. Ach-evoked $Ca^{2+}$ oscillations did not arise in the absence of external $Ca^{2+}$ but rapidly resumed on $Ca^{2+}$ restitution to the bath. However, nicotine, a selective agonist of the $Ca^{2+}$-permeable nicotinic receptors, did not cause any detectable increase in $[Ca^{2+}]_i$. Pharmacological manipulation indeed revealed that Ach-induced $Ca^{2+}$ spikes in bEnd5 cells are triggered by the interaction between intracellular $Ca^{2+}$ release from $InsP_3$ receptors ($InsP_3Rs$) SOCE. SOCE was then amplified by $Ca^{2+}$ release through $InsP_3Rs$ and ryanodine receptors (RyRs), thereby shaping the $Ca^{2+}$ spikes. Consistently, the pharmacological depletion of the ER $Ca^{2+}$ store with cyclopiazonic acid (CPA), a selective inhibitor of Sarco-Endoplasmic Reticulum $Ca^{2+}$-ATPase (SERCA), revealed the expression of a BTP2- and $La^{3+}$-sensitive SOCE in bEnd5 cells. Next, we found that Ach-induced NO production was hindered by L-NAME, a selective NOS inhibitor, and BAPTA, a membrane permeable intracellular $Ca^{2+}$ buffer. Moreover, Ach-elicited NO synthesis was blocked by the pharmacological abrogation of the accompanying $Ca^{2+}$ spikes.

**Conclusions:** Ach stimulates bEnd5 cells by inducing a burst of intracellular $Ca^{2+}$ spikes which is patterned by the interplay between ER-dependent $Ca^{2+}$ mobilization and SOCE. Ach-elicited $Ca^{2+}$ spikes result in NO production and are, therefore, predicted to control local CBF in mouse brain. Future experiments will assess whether this signaling pathway is altered in neurodegenerative disorders, such as Alzheimer's disease.

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## Acidic $Ca^{2+}$ stores interact with the endoplasmic reticulum to shape intracellular $Ca^{2+}$ signals in human endothelial progenitor cells

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**Objectives:** Most cellular processes are finely regulated by changes in intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$. The endoplasmic reticulum (ER) represents the main intracellular $Ca^{2+}$ reservoir and mobilizes intraluminally stored $Ca^{2+}$ by activating either the inositol-1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$) and/or the ryanodine receptors (RyRs). Acidic organelles of the endolysosomal (EL) system constitute an additional endogenous $Ca^{2+}$ pool which is mobilized upon the activation of the so-called two-pore channels (TPCs) by nicotinic acid adenine dinucleotide phosphate (NAADP). A growing number of studies showed that ER and EL $Ca^{2+}$ stores may establish a functional cross-talk to regulate intracellular $Ca^{2+}$ signals and $Ca^{2+}$-dependent processes in many cell types. Herein, we sought to assess the functional coupling between ER and EL $Ca^{2+}$ stores in circulating human endothelial progenitor cells (hEPCs), which promote revascularization of ischemic tissues and favor the angiogenic switch in growing tumors.

**Materials and methods:** Changes in $[Ca^{2+}]_i$ were monitored from hEPCs loaded with the $Ca^{2+}$-sensitive dye, Fura-2/AM (2 μM, 30 min), by using a CCD camera.

**Results:** The lysosomotropic agent GPN caused a sustained increase in $[Ca^{2+}]_i$ which was unaffected by removal of external $Ca^{2+}$ (0 $Ca^{2+}$), thereby indicating that EL $Ca^{2+}$ stores liberate $Ca^{2+}$ without activating a concomitant $Ca^{2+}$ inflow in hEPCs. Consistently, bafilomycin A1 (Baf), which collapses the pH gradient (ΔpH) necessary for $Ca^{2+}$ uptake into acidic stores, elevated $[Ca^{2+}]_i$ in 0 $Ca^{2+}$. Subsequently, we found that GPN, Baf and nigericin, which also dissipates the ΔpH of acidic organelles, significantly ($p < 0.05$) reduced the $Ca^{2+}$ response to cyclopiazonic acid (CPA), a selective inhibitor of ER $Ca^{2+}$ sequestration. In turn, CPA dampened both GPN- and Baf-induced $Ca^{2+}$ mobilization. Moreover, GPN-evoked $Ca^{2+}$ signals were fully inhibited by tetracaine and curtailed by 2-APB, which inhibit RyRs and $InsP_3Rs$, respectively. Overall, these data indicate that EL-dependent $Ca^{2+}$ mobilization stimulates $Ca^{2+}$ mobilization through ER-embedded $InsP_3Rs$ and RyRs through the process of $Ca^{2+}$-induced $Ca^{2+}$ release (CICR). Finally, we found that TPC1 and TPC2 are both expressed in hEPCs and that Ned-19, a selective blocker of NAADP-induced $Ca^{2+}$ release, blocks the $Ca^{2+}$ response to ATP.

**Conclusions:** These results suggest that $Ca^{2+}$ release from EL stores induces ER-dependent $Ca^{2+}$ mobilization through the process of CICR in hEPCs. This interaction leads to NAADP-mediated physiological $Ca^{2+}$ signals and builds a new level of complexity to our understanding of control of cellular $Ca^{2+}$ homeostasis in hEPCs.

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