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Hypoxia-Induced Sarcoplasmic Reticulum Ca^{2+} Leak Is Reversed by Ryanodine Receptor Stabilizer JTV-519 in HL-1 Cardiomyocytes

ORIGINAL INVESTIGATION

ABSTRACT

Background: To assess whether hypoxia, as can be found in obstructive sleep apnea syndrome, is causally associated with the development of heart failure through a direct effect on calcium leakage from the sarcoplasmic reticulum.

Methods: The impact of hypoxia on sarcoplasmic reticulum calcium leakage and expression of RyR2 (ryanodine receptor2) and SERC2a (sarcoplasmic reticulum Ca^{2+}ATPase 2a) was investigated together with the outcomes of JTV-519 and S107 treatment. HL-1 cardiomyocytes were cultured for 7 days on gas-permeable cultureware under control (12% O_2) or hypoxic (1% O_2) conditions with or without JTV-519 or S107. SRCL was assessed using a Fluo-5N probe. Gene and protein expression was analyzed using qPCR and western blotting.

Results: Hypoxic exposure increased sarcoplasmic reticulum calcium leakage by 39% and reduced *RyR2* gene expression by 52%. No effect on RyR2 protein expression was observed. Treatment with 1μM JTV-519 reduced sarcoplasmic reticulum calcium leakage by 52% and 35% under control and hypoxic conditions, respectively. Administration of 1 μM JTV-519 increased *RyR2* gene expression by 89% in control conditions. No effect on SRCL, RyR2, or SERC2a gene, or protein expression was observed with S107 treatment.

Conclusion: Hypoxia increased sarcoplasmic reticulum calcium leakage which was ameliorated by JTV-519 treatment independently of gene or protein expression. JTV-519 represents a possible treatment for obstructive sleep apnea-associated HF.

Keywords: Calcium, heart failure, obstructive sleep apnea, hypoxia, ryanodine receptor

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INTRODUCTION

Obstructive sleep apnea (OSA) is a common condition affecting 936 million individuals aged 30–69 years worldwide.[1] OSA is characterized by repetitive cessation of inspiratory airflow during sleep, exposing the whole body to repetitive hypoxic episodes. Besides the negative impact on sleep structure, quality of life, and metabolic health, OSA is associated with increased overall and cardiovascular mortality and has been suggested to play a causal role in the pathogenesis of atherosclerosis, hypertension, and arrhythmias.[2] Furthermore, epidemiological evidence identified OSA as an independent risk factor that increases the risk of developing heart failure more than 2-fold.[2,3] Molecular mechanisms mediating this association are poorly understood.

Heart failure, a clinical syndrome characterized by an inability of the heart to generate sufficient cardiac output for the metabolic needs of the body, represents a significant cause of mortality and morbidity with a rapidly increasing prevalence. It currently affects approximately 26 million people worldwide[4] and up to 53% of patients diagnosed with heart failure also suffer from moderate to severe OSA.[5] The presence of heart failure increases the severity of OSA through the fluid redistribution from legs followed by parapharyngeal soft tissue edema and airway narrowing in the horizontal position. Current research suggests that OSA could promote the development of heart failure via multiple mechanisms. For example, repetitive chest expansions against an obstructed upper airway (Müller's

maneuver) generate excessive negative inspiratory intrathoracic pressure with a subsequent increase in right ventricular preload and left ventricular afterload.[5] Additionally, fragmented sleep with persistently enhanced sympathetic nerve activity inducing peripheral vasoconstriction and β-adrenoreceptor desensitization[5,6] represent key features linking OSA with the development of heart failure. OSA patients often exhibit endocrine and inflammatory pathways dysregulation such as elevated corticoid levels and the activation of the renin-angiotensin-aldosterone system.[7] This dysregulation, combined with the generation of reactive oxygen species, is also linked to the pathogenesis of heart failure.[5]

Investigation of cellular and molecular derangements of heart failure reveals a critical role of intracellular Ca^{2+} handling in the heart. Cardiomyocytes in heart failure patients exhibit increased resting Ca^{2+} levels in the cytoplasm combined with decreased depolarization-triggered Ca^{2+} transient amplitudes.[8] This constellation of factors is associated with deteriorated excitation-contraction coupling and reduced myocardial contractile forces, representing key features of a failing heart. Molecular mechanisms, identified as key factors contributing to heart failure, include the diastolic leakage of Ca^{2+} through ryanodine receptors (RyR2) (found on the sarcoplasmic reticulum membrane and mediating Ca^{2+} efflux after depolarization), together with the decreased diastolic activity of sarcoplasmic reticulum Ca^{2+}ATPase 2a (SERCA2a) (found on the sarcoplasmic reticulum membrane and securing re-uptake of Ca^{2+} back into the sarcoplasmic reticulum after contraction).[8,9] Novel pharmacological approaches using ryanodine receptor stabilizers (Rycals) have been developed to correct the impaired Ca^{2+} metabolism in failing cardiomyocytes.[10] It was reported previously that hypoxia modulates the expression and function of calcium channels and impacts intracellular Ca^{2+} handling, for example, in the context of acute ischemia-reperfusion injury.[11] However, it remains unknown whether similar effects (including the beneficial outcomes with Rycals) would be observed after chronic and/or repetitive exposure to hypoxia, specifically in the context of heart failure potentially induced by OSA.

In order to investigate the contribution of hypoxia (a hallmark of OSA) independent from concurrent factors contributing to the pathogenesis of heart failure (e.g., metabolic, endocrine, neuronal, and mechanical causes), we decided to study HL-1 cardiomyocytes exposed in-vitro to defined O_2 concentrations, employing a validated system for hypoxic cell exposures using membrane-bottom cultureware.[12] The HL-1 cardiomyocytes cell line can be serially passaged without losing differentiated phenotype and retain structural and functional features, enabling proper response to pharmacological stimuli.[13] Furthermore, the in-vitro approach allows for performing longer hypoxic exposures (7 days) than possible with isolated cardiomyocytes.

The aim of the study was to assess whether hypoxia as occurs, for example, in OSA syndrome is causally associated with the development of heart failure through a direct effect on intracellular calcium metabolism via sarcoplasmic reticulum calcium leak. In order to study this relationship, the effect of 1,4-benzothiazepine derivates (Rycals: JTV-519 and S107) on intracellular Ca^{2+} handling as well as protein and gene expression of RyR2 and SERCA2a under hypoxic conditions was investigated.

HIGHLIGHTS

- Hypoxia leads to an impairment in intracellular calcium metabolism in cardiomyocytes, independent of endocrine and neuronal mechanisms, and contributes to the development of heart failure as exemplified in patients with obstructive sleep apnea.
- Our study results show increased calcium leak through the cardiac ryanodine receptor (RyR2) after exposure to hypoxia, and this condition was reversed by adding JTV-519 to the cultivation medium. RyR2 gene expression was also increased after JTV-519 exposure but this effect was lost in hypoxia.

METHODS

Cell Culture

HL-1 cardiomyocytes (SCC065, Sigma-Aldrich, St. Louis, Mo, USA) were cultured as previously described.[13] Cells were grown in Claycomb medium enriched with fetal bovine serum 10%, penicillin/streptomycin 100 U/mL:100 µg/mL, 0.1 mM norepinephrine, and 2 mM L-glutamine in T25 flasks (C6481, Sigma-Aldrich). After reaching confluence, they were harvested and plated on 50-mm diameter fluorocarbon-bottom dishes, which are highly permeable for gases for hypoxic exposure (Lumox, Sarstedt AG&Co, Nümbrecht, Germany).

Hypoxic Experiments and Treatment with Chemicals (Rycals)

For hypoxic exposures, dishes were placed in the modular chamber incubators (Billups-Rothenberg Inc., Del Mar, Calif, USA) and flushed with calibration quality gas mixtures of 12% O_2 + 5% CO_2 or 1% O_2 + 5% CO_2 (Linde Gas a.s., Prague, Czech Republic) to achieve control and hypoxic exposures, respectively. The concentration of 12% O_2 for control experiments was selected based on 2 factors. First, in the atmospheric air the partial pressure of O_2 is 160 mm Hg (~ 21% O_2). During inspiration, this air is humidified and mixed with exhaled CO_2 resulting in a composition of alveolar gas with an O_2 partial pressure of 100 mm Hg (~ 13%). Second, previous experiments measuring oxygen levels directly in the myocardium showed the highest O_2 level of 94–96 mm Hg (~12%).[14]

Drugs JTV-519 and S107 are 1,4-benzothiazepine derivatives that stabilize the closed state of RyR2 proteins by increasing their affinity to calstabin 2 (by conformational change[15]) leading to reduced diastolic Ca^{2+} SR depletion.[16,17] Rycals JTV-519 (SML0549, Sigma-Aldrich) and S107 (5.00469, Sigma-Aldrich) or Vehicle (DMSO, 276855, Sigma-Aldrich) were added to culture media as appropriate to achieve 0.3 µM and 1 µM for JTV-519 and 0.1 µM and 10 µM for S107. Concentrations of JTV-519 and S107 were selected based on previously published in-vitro experiments.[18,19]

To insure the longest hypoxic exposure possible, mimicking chronic hypoxia (e.g., in the context of OSA), matching HL-1 growth kinetics, and preventing them from overgrowth, cells were incubated in modular chambers for 7 days, the medium was changed every 48 hours, and cells were then used for the experiments described below.

Gene Expression Analysis

The RNA was isolated using the High Pure RNA Isolation Kit (11828665001, Roche Diagnostics, Basel, Switzerland). The quality of isolated RNA was verified using spectrophotometric methods and a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Del, USA). Only samples with an absorbance ratio of 260 nm/280 nm greater than 2 were used. Extracted RNA was then transcribed using high-capacity cDNA Reverse Transcription Kit (4368814, Roche Diagnostics) and gene expression of *RyR2* (ryanodine receptor 2), *SERCA2a* (sarcoplasmic reticulum Ca^{2+}ATPase 2a), *TBP* (TATA box binding protein), *GUSB* (glucuronidase beta) was assessed using Applied Biosystem 7500 Fast Real-Time PCR (Applied Biosystems, Foster City, Calif, USA) using TaqMan probes (Prod. ID: Mm00465877_m1, Mm01201431_m1, Mm00446971_m1, and Mm01197698_m1). *TBP* and *GUSB* were used as endogenous controls. Data are presented as relative gene expression changes comparing target genes to endogenous controls ($2^{-\Delta Ct}$).

Protein Expression Analysis

The cells were lysed by adding 0.5 mL of T-PER Tissue Protein Extraction Reagent (78510, Thermo Fisher Scientific) and centrifuged (12,000 rpm, 4°C, 15 minutes). The supernatant was mixed with Laemli buffer (161-0737, Bio-Rad Laboratories, Hercules, Calif, USA) and proteins were separated using SDS-PAGE electrophoresis in a 10% gel for SERCA2a and a gradient gel (4%–12%) for RyR2. Next, they were blotted on a 0.2 μM polyvinylidene fluoride membrane for 1.5 hours at 100 V, or overnight at 30 V and 2 hours at 100 V, respectively, in cooled Transfer buffer (2317, Bio-Rad Laboratories). The membranes were blocked using EveryBlot Blocking Buffer (12010020, Bio-Rad Laboratories, Hercules, CA, USA) for 5 minutes. After blocking, the membranes were washed using Tris-buffered saline with Tween (TBS-T) and then incubated with primary antibody SERCA2a (A010-23S, Badrilla, Leeds, UK, dilution 1:1000), RyR2 (ARR-002, Alomone Labs, Jerusalem, Israel, dilution 1:1000) and β-tubulin (ab6046, Abcam, Cambridge, UK, dilution 1:1000) overnight. The next morning the membranes were washed with TBS-T and incubated for 1 hour with secondary antibody goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (sc-2004, Santa Cruz Biotechnology, Dallas, Tex, USA, dilution 1:20000). Band detection was performed using Radiance Plus Chemiluminescent Substrate (Azure Biosystems, Dublin, Calif, USA) and digitalized using the ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules). Band intensities were quantified by Image Lab Software (Bio-Rad Laboratories, Hercules) and normalized to β-tubulin.

Sarcoplasmic Reticulum Ca^{2+} Leak Measurement

Cells were plated on glass coverslips and cultured as described above. After 7 days of exposures, cells were loaded with the low calcium affinity probe Fluo-5N, AM (4 μM) together with 0.02% Pluronic F-127 (P6867, Thermo Fisher Scientific) for 1 hour at 37°C. The coverslips were then mounted in an RC-30 confocal imaging chamber (Warner Instruments, Holliston, Mass, USA) for live-cell imaging. Subsequently, cells were continually perfused with 0.3 mL/minute of Hank's balanced salt solution (14025100, Thermo Fisher Scientific) and heated to 37°C using an inline solution heater (SH-27B, Warner Instruments). After 5 minutes of initial perfusion, cells were perfused with HBSS containing 10 μM thapsigargin (T9030, Sigma-Aldrich) to block Ca^{2+} re-uptake by SERCA2a, which is shown in Figure 1. The decay of fluorescence emission intensity at 516 nm (representing Ca^{2+} leakage through RyR2) was measured after excitation with an argon-ion laser at 488 nm using a Leica TCS SP5 confocal microscope (20× objective, Leica Microsystems, Manheim, Germany). Representative example is shown in Figure 2. An exponential decay function was fitted to the data ($y = A0e - kt$), and key parameters, the rate constant (k), half-life, and time constant, were then calculated.

Statistical Analysis

To test the distribution of collected data, the Kolmogorov–Smirnov and Shapiro–Wilk normality tests were employed and showed normal distribution of all variables with the exception of sarcoplasmic reticulum Ca^{2+} leak, where Kolmogorov–Smirnov test could not be calculated (while Shapiro-Wilk showed normal distribution of data) and thus, for the sake of statistical conservativism, non-parametric test (Mann–Whitney U) results were reported as well. The effect of hypoxia on the studied variables was assessed using the *t*-test. Two-way ANOVA with Tukey post hoc test was employed to assess the independent effects of hypoxia, pharmacological treatment, and their interaction using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). Six replications were performed for each group for gene and protein expression, and 4 replications were performed for control

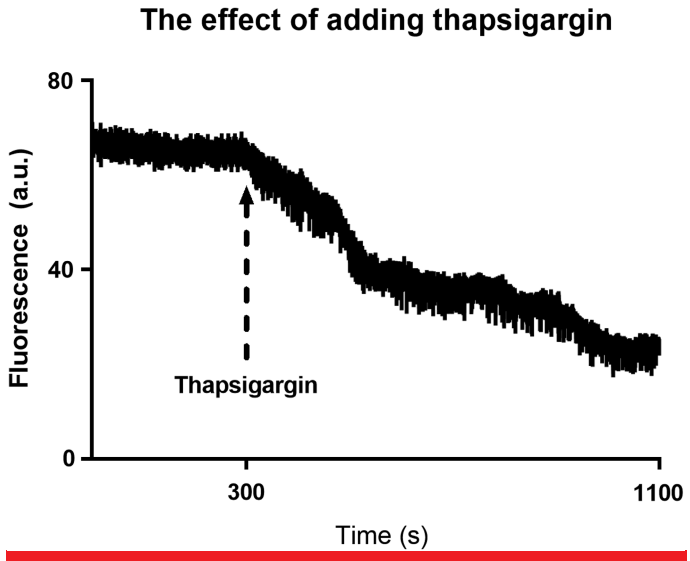

Figure 1. The effect of SERCA2a blockade with thapsigargin. Representative example of Ca^{+2} levels recording (fluorescence of Fluo-5N) after adding thapsigargin to the HL-1 cardiomyocytes.

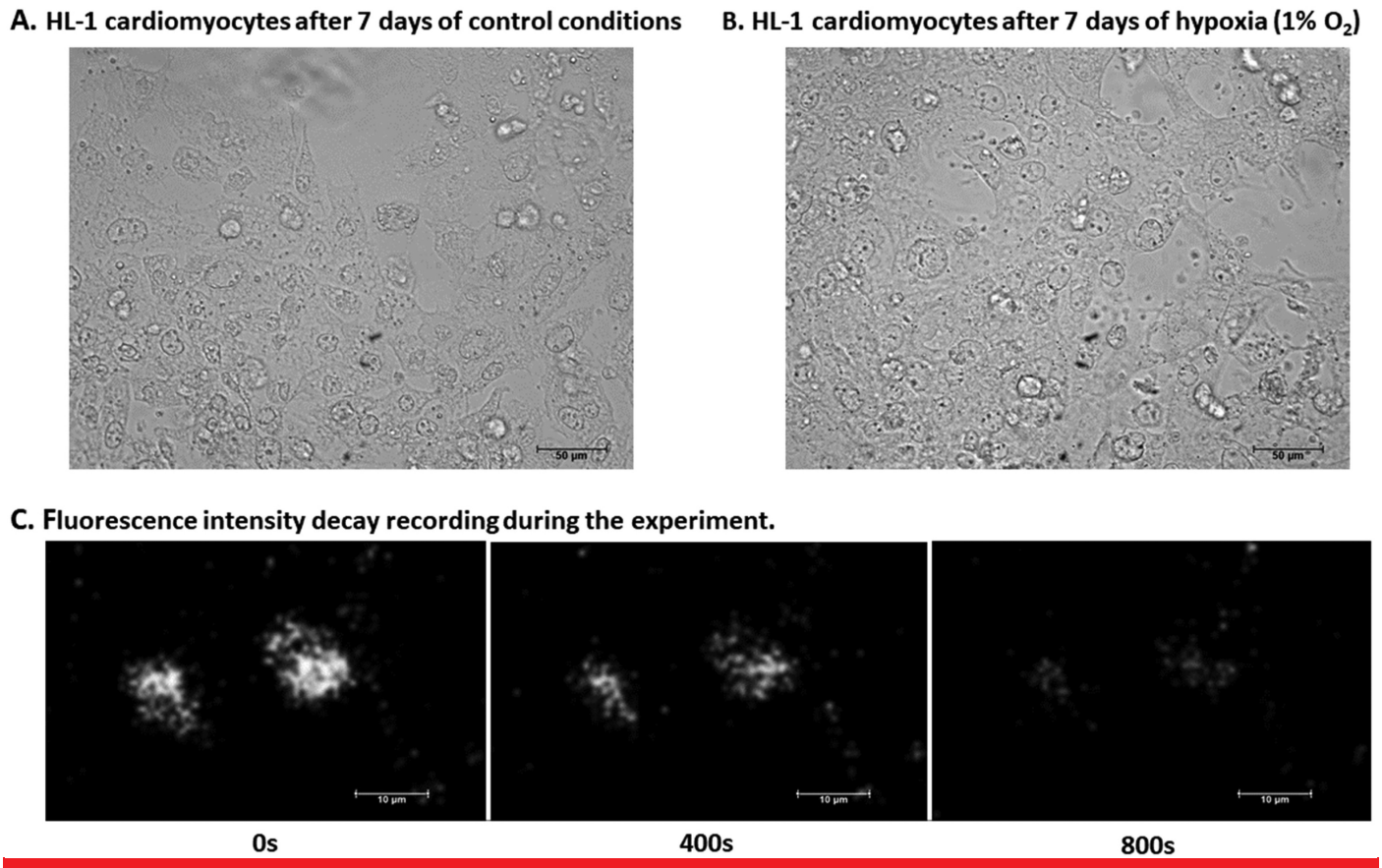

Figure 2. HL-1 cardiomyocytes after seven days of hypoxia/control conditions and fluorescence time lapse. (A) Bright-field image of HL-1 cardiomyocytes after 7 days of hypoxia (1% O_2). (B) Bright-field image of HL-1 cardiomyocytes after 7 days of control conditions (12% O_2). (C) Representative example of fluorescence intensity decay recoding during the experiment.

and 3 for exposure groups in sarcoplasmic Ca^{2+} leak measurements. The statistical significance was set as $P < .050$ in all tests. Data are presented as the mean ± SD.

RESULTS

The Effect of Hypoxia and Rycals on Gene Expression

Exposure to hypoxia (1% O_2) reduced *RyR2* gene expression by 52% (0.37 ± 0.16 vs. 0.77 ± 0.14, $P < .001$), while *SERCA2a* gene expression remained unaffected (12.13 ± 1.43 vs. 11.44 ± 2.32, $P = .393$). The effect of JTV-519 treatment was only observed under control conditions, where *RyR2* gene expression increased by 89% (1.40 ± 0.36 vs. 0.74 ± 0.12, $P < .001$), following treatment with 1 μM JTV-519. No effect of JTV-519 on *RyR2* gene expression was observed under 1% O_2 exposure (0.30 ± 0.03 vs. 0.33 ± 0.07, $P = .426$) and (0.28 ± 0.03 vs. 0.33 ± 0.07, $P = .156$), respectively. As a result, hypoxia-induced reduction in *RyR2* gene expression was unaffected by JTV-519 treatment. Data are summarized in Figure 3A. Adding 10 μM of S107 to the culture media had a positive effect on *RyR2* gene expression, but did not reach the statistical significance. Gene expression of *RyR2* was increased by 90% (1.52 ± 0.87 vs. 0.80 ± 0.16, $P = .073$) only under control conditions (12% O_2) but no effect was observed under hypoxic conditions (1% O_2), where *RyR2* gene expression remained unchanged by S107 (0.41 ± 0.22 vs 0.34 ± 0.07, $P = .487$) and (0.41 ± 0.22 vs 0.29 ± 0.07, $P = .221$), respectively. Data are summarized in Figure 3B. *SERCA2a* gene expression was not affected by JTV-519 under control conditions but was decreased by 32% under 1% O_2 conditions (8.41 ± 0.55 vs 12.30 ± 1.11, $P < .001$). Treatment with S107 had no effect on *SERCA2a* expression under 12% O_2 or 1% O_2 conditions. Data are summarized in Figure 3C and 3D.

The Effect of Hypoxia and Rycals on Protein Expression

SERCA2a and RyR2 protein expression was unchanged by exposure to 1% O_2 (1.99 ± 0.74 vs. 1.49 ± 0.62, $P = .088$) and (0.88 ± 0.49 vs. 0.87 ± 0.09, $P = .968$), respectively. Treatment with JTV-519 had n effect on protein expression of SERCA2a (2.06 ± 1.43 vs. 1.45 ± 1.17, $P = 0.435$) or RyR2 (0.89 ± 0.36 vs. 0.59 ± 0.22, $P = .145$). Likewise, S107 had no effect on protein expression of SERCA2a and RyR2 under control (12% O_2) or hypoxic (1% O_2) conditions (1.43 ± 0.30 vs. 1.64 ± 0.72, $P = .519$) and (0.77 ± 0.25 vs. 0.71 ± 0.16, $P = .592$), respectively. Data are summarized in Figures 4A-4D. Analysis of protein concentration using BCA assay showed significant decrease of protein levels in control conditions compared to hypoxia (4.80 ± 0.58 vs. 2.60 ± 0.20 mg/mL, $P < .001$).

Sarcoplasmic Reticulum Ca^{2+} Leak

Exposure of HL-1 cardiomyocytes to 1% O_2 (hypoxia) increased Ca^{2+} leak from sarcoplasmic reticulum by 39% as compared to control (12% O_2) conditions (Half-life: 256.7 ± 5.6 s vs. 420.5 ±

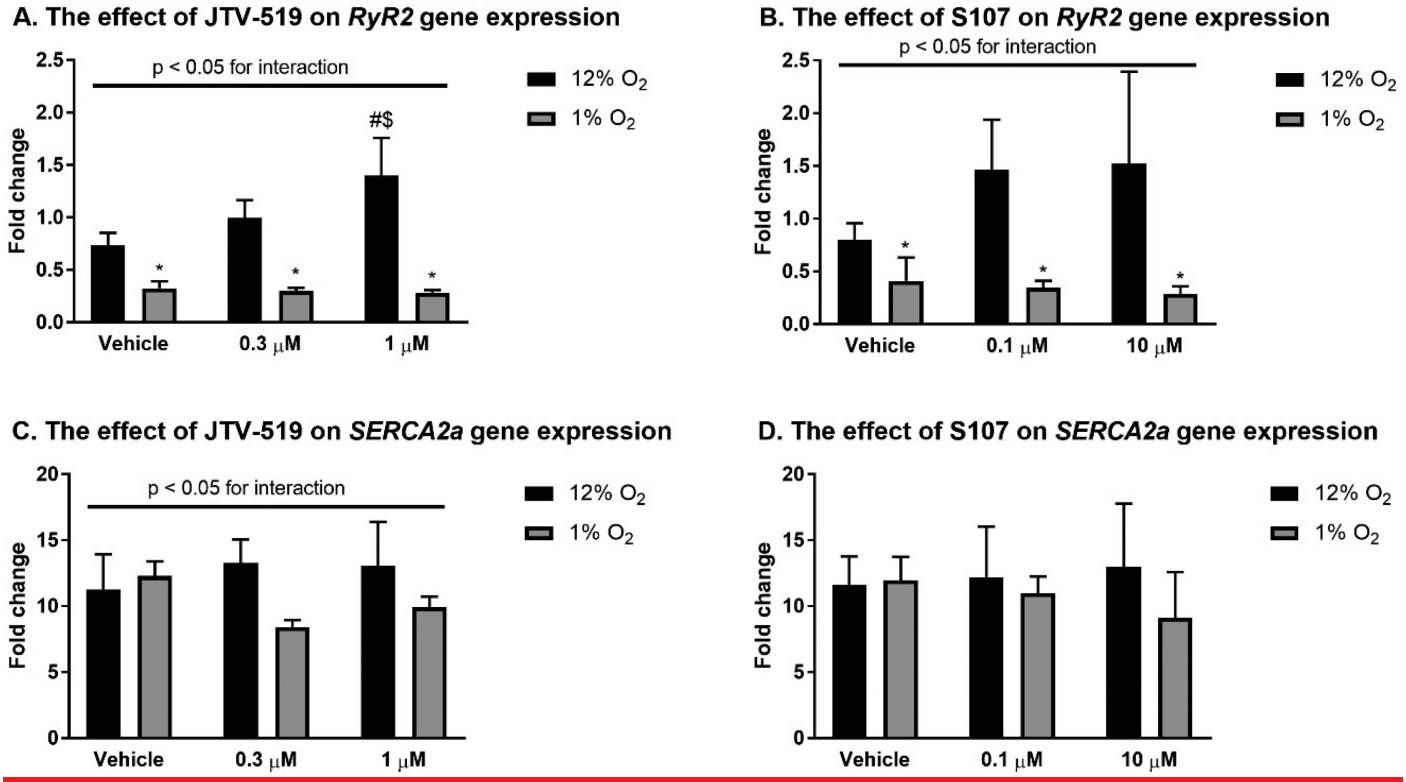

Figure 3. The effect of hypoxia and Rycal treatment on RyR2 and SERCA2a gene expression. The effect of 7-day hypoxic and normoxic exposure with Rycals/Vehicle (DMSO) treatment on RyR2 and SERCA2a gene expression. n = 6, * P < .050 for comparison with 1% O_2, # P < .050 for comparison with Vehicle, $ P < .050 for comparison with 0.3 mM (all 1-way ANOVA with Tukey post hoc test).

21.6 s, $P = .025$). Treatment of cells with 1 μM JTV-519 throughout exposures significantly reduced Ca^{2+} leak from sarcoplasmic reticulum under both control and hypoxic conditions by 52% (Half-life: 420.5 s ± 21.6 vs 867.8 s ± 84.2, $P = .005$), and 35% (Half-life: 256.7 s ± 5.6 vs 396.4 s ± 17.4, $P = .026$), respectively. In fact, 1 μM JTV-519 treatment normalized hypoxia-induced Ca^{2+} leak from sarcoplasmic reticulum to levels observed under control (non-hypoxic) conditions (Half-life: 420.5 ± 21.6 s vs. 396.4 ± 17.4 s, $P = .284$). As S107 showed no significant effect on gene or on protein expression, sarcoplasmic reticulum Ca^{2+} leak experiments were not performed for this drug. Data are summarized in Table 1 including appropriate rate and time constants. Representative examples of sarcoplasmic reticulum Ca^{2+} leak are presented in Figures 5A-5B.

DISCUSSION

In the presented study, we observed that HL-1 cardiomyocytes exposed to 7 days of hypoxia (1% O_2) exhibit increased Ca^{2+} leakage from the sarcoplasmic reticulum, which was then normalized by treatment with the novel drug, JTV-519, during exposure. Additionally, we observed that gene expression of *RyR2* (but not *SERCA2a*) was reduced by hypoxia, while protein expression of both proteins remained unchanged. It was also observed that JTV-519 had a different effect on *RyR2* gene expression; under control conditions, there was increased gene expression whereas no change was observed under hypoxic conditions. Our investigation provides a molecular background for the possible use of JTV-519 as a therapeutic intervention for OSA-induced heart failure.

Effective excitation-contraction coupling is secured by the interaction between membrane depolarization and intracellular calcium handling. During physiological conditions, membrane depolarization activates L-type Ca^{2+} channels followed by Ca^{2+} flux into cardiomyocytes. Ca^{2+} ions then diffuse through junctional zones and bind to (activate) ryanodine receptors (RyR2), mediating a massive Ca^{2+} efflux from the sarcoplasmic reticulum (SR) into the cytoplasm. Ca^{2+} subsequently binds to troponin-C and actin-myosin cross-bridging follows. The cycle ends when calcium is transported back into the SR via SR Ca^{2+}ATPase (SERCA2a) and out of the cell via Na^{+}-Ca^{2+} exchangers.[9] Our study suggests that hypoxia could be 1 of the causal factors linking the development of heart failure with OSA.

Exposure of HL-1 cells to hypoxia (1% O_2) increased calcium leakage from the SR, thus reducing cytoplasmic depolarization-induced Ca^{2+} transient and strength of contraction. In particular, Ca^{2+} leakage from the SR is recognized as 1 of the key mechanisms driving the pathogenesis and progression of heart failure.[4,20] The literature has shown that the direct effect of hypoxia on RyR2 function could be (at least partially) mediated by redox-active reagents since RyR2 exhibit high sensitivity to oxidation and nitrosylation.[21,22] Exposure to reactive oxygen/nitrogen species is also found to increase the RyR2 open probability[23,24] and reduce the binding of regulatory proteins (e.g., calstabin[25–27]) to RyRs. The function

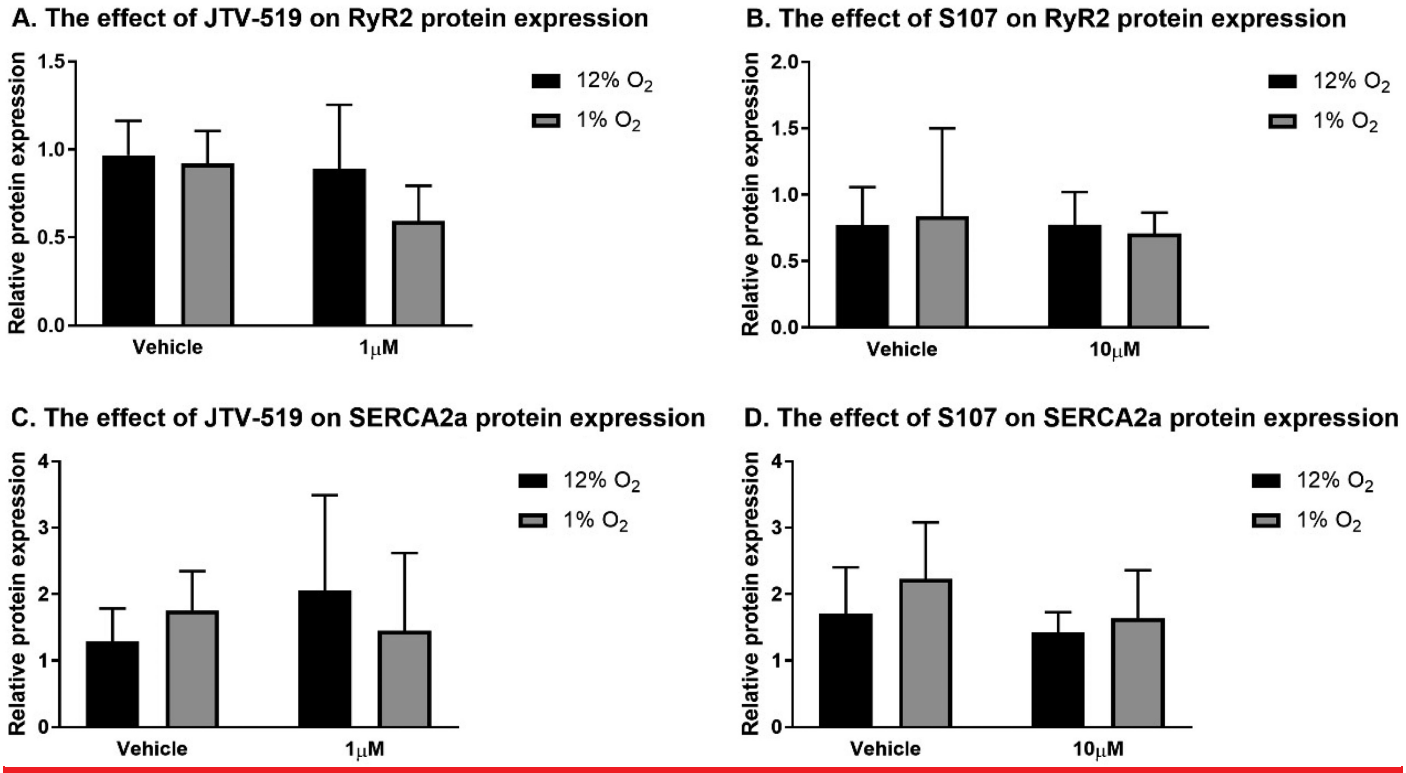

Figure 4. The effect of hypoxia and Rycal treatment on RyR2 and SERCA2a protein expression. The effect of 7-day hypoxic and normoxic exposure with Rycals/Vehicle (DMSO) treatment on RyR2 and SERCA2a protein expression. n = 6. No statistically significant comparison.

of RyR2 proteins is also subject to extensive regulation by phosphorylation through the activity of PKA (protein kinase A) and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II).[28] Hypoxia was reported to increase the cardiomyocyte activity of PKA as well as CaMKII, in-vivo[29] and in-vitro.[30] It has also been reported that aberrant RyR2 phosphorylation augments dissociation of the RyR2 channel complex and promotes Ca^{2+} leakage. Specifically, serine phosphorylation at particular positions (e.g., S2808, S2030, S2814, and S2811) are suggested to play a key role in the development of heart failure.[28,31,32] Hypoxia-promoted RyR2 phosphorylation represents another molecular explanation for increased sarcoplasmic Ca^{2+} leakage, as observed in our study.

Treatment with novel drug JTV-519, a novel drug, which prevents dissociation of calstabin from RyR2 and inhibits the RyR2 complex dissociation and Ca^{2+} leak,[15] proved to be effective in reducing Ca^{2+} leakage under control as well as hypoxic conditions in our study. Administration of JTV-519 normalized SR Ca^{2+} leakage from hypoxic cells to levels observed under control conditions without changes in gene or protein expression. This suggests a direct interaction between JTV-519 and RyR2 channels. Proving the effectiveness of JTV-519 under hypoxic conditions prepares the ground for possible pharmacological treatment and/or prevention of OSA-induced heart failure. This novel discovery is significant when considering standard OSA treatment modalities, that is, continuous positive airway pressure for obstructive sleep apnea or adaptive servo-ventilation for central sleep apnea has shown no cardiovascular benefit and can even worsen cardiovascular outcomes.[33,34] Furthermore, it has been documented that 25–73% of OSA subjects indicated for CPAP treatment do not adhere sufficiently to the treatment.[35]

Table 1. Sarcoplasmic Reticulum Ca^{2+} Leak

	Vehicle		JTV-519 (1 μM)	
	12% O_2	1% O_2	12% O_2	1% O_2
K*10^{-4} (1/s)	1.65 (0.08) *$	2.70 (0.06)	0.80 (0.08) *#$‡	1.75 (0.08) #‡
Half-life (s)	420.5 (21.6) *$	256.7 (5.6)	867.8 (84.2) *#$‡	396.4 (17.4) #‡
Tau (s)	606.6 (31.1) *$	370.4 (8.0)	1252.0 (121.4) *#$‡	571.9 (25.2) #‡

Data are mean (SD), K = rate constant, Tau = time constant.
*Significant difference compared with 1% O_2 group (P = .025 for Vehicle, P = .042 for JTV-519) using 1-way ANOVA with Tukey post-hoc test
#Significant difference compared with Vehicle (P = .005 for 12%, P = .026 for 1%) using 1-way ANOVA with Tukey post-hoc test
$Significant difference compared with 1% O_2 group (P = .034 for Vehicle, P = .034 for JTV-519) using Mann–Whitney U test
‡Significant difference compared with Vehicle (P = .021 for 12%, P = .021 for 1%) using Mann–Whitney U test n = 4 for Vehicle and n = 3 for JTV-519 (1 μM).

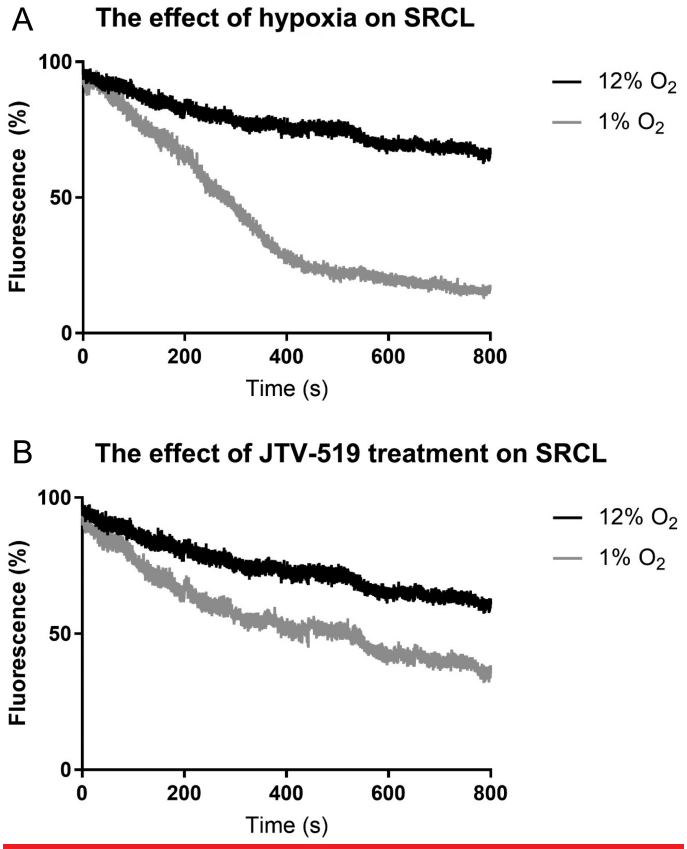

Figure 5. The effect of hypoxia and JTV-519 treatment on sarcoplasmic reticulum Ca^{2+} leak. Representative examples of sarcoplasmic reticulum Ca^{+2} levels after thapsigargin administration (A) and the effect of hypoxia, (B) the effect of adding JTV-519 to the cultivation medium on SRCL (sarcoplasmic reticulum calcium leak).

Gene expression of *RyR2* was markedly reduced under hypoxic conditions in our study, which is in line with reduced *RyR2* gene expression observed in heart failure patients[36] and in rodent models of heart failure.[37] Despite the importance of *RyR2* in intracellular calcium metabolism and the fact that the regulatory region of the *RyR2* gene was characterized in 1996,[38] little information is available on the regulation of *RyR2* gene expression in the literature. It has been demonstrated that severe food restriction down-regulates *RyR2* gene expression[39] and that thyroxine treatment up-regulated *RyR2* expression[37] in the heart, suggesting a link between *RyR2* gene expression and the overall metabolic status of the cardiomyocyte. Interestingly, RyR2 mRNA levels as well as RyR2 function were found to be directly regulated by circadian rhythm proteins (e.g., clock protein) in the suprachiasmatic nucleus.[40]

One of the most intriguing and novel findings of our study is the observation that *RyR2* gene expression was up-regulated by the administration of JTV-519 in a dose-response manner under control conditions (12% O_2). Previously, JTV-519 was shown to activate cardiac PKC-δ (protein kinase C-δ) mediating preconditioning-like heart protection.[41] In parallel, PKC isoforms were found to be strong regulators of gene expression,[42] and it is thus plausible to hypothesize that gene expression regulation by JTV-519 (mediated by PKC-δ) represents an additional effect of JTV-519 on intracellular Ca^{2+} metabolism, beyond the stabilization of RyR2. Future studies are needed to validate this theory and to explore the absent effect of JTV-519 on *RyR2* gene expression under hypoxic conditions.

In contrast, RyR2 and SERCA2a protein expression remained unchanged by hypoxic exposure or pharmacological treatments suggesting independent regulations of gene and protein expressions.[43] For example, excessive protein production might subsequently reduce its gene expression in a negative feedback loop mediated, for example, by RNA/protein interactions or microRNA.[44,45] Additionally, previous studies showed that hypoxia reduced protein translation in a dose-response manner.[46] Concurrently, increased cytosolic calcium levels (due to RyR2 Ca2+ leakage) stimulate calmodulin-dependent kinase-β with subsequent activation of AMP-activated protein kinase and inhibition of mTOR (mammalian target of rapamycin), ultimately inducing cellular autophagy and preventing proteins levels from increasing.[47]

Study Limitations

Conducting experiments using an in-vitro cell line enabled the investigation of the separate effects of pericellular oxygen concentrations on key features of Ca^{2+} metabolism, without the interfering influence of other factors induced by hypoxia in the whole organism (e.g., circulatory, neuronal, and endocrine changes[48,49]). However, these advantages become disadvantages when results are to be extrapolated to animal or human pathophysiology. Although we employed the HL-1 cell line that is characterized by morphological, biochemical, and electrophysiological characteristics of differentiated cardiomyocytes,[13] it is essential to note limitations associated with the experimental approach. First, the degree of hypoxia used in the study was more profound than what is observed in sleep apnea syndrome, and the duration of exposure was significantly shorter that in the context of human heart failure. Second, using special fluorocarbon-bottom plates enabled exposure of cells to specific O_2 levels; however, the physical and chemical properties of culture surfaces differed from standard plastic cultureware.[50]

Third, measuring Ca^{2+} sarcoplasmic leakage with a Fluo-5N, a single wavelength fluorescence probe, has 2 important limitations: (a) it does not allow for intracellular Ca^{2+} quantification in absolute units (nmol/L). Thus, data are expressed and presented as relative fluorescence intensity over time and (b) inter-cellular differences in fluorescence signal intensity are rather high due to uneven cell loading and distribution and require the expression of data as relative values (% of baseline). Fourth, hypoxia has been shown to reduce cell proliferation in various cell types,[51] and our data similarly shows that cell quantity is decreased in hypoxia. However, it should be noted that the HL-1 cardiomyocytes are fully differentiated[13] and thus their phenotype (displayed at Figure 2) should not be affected by reduced proliferation." To minimalize the potential influence of different

cellular growth, all data were normalized to protein concentration (western blotting) or cDNA concentration (qPCR). Additionally, single cell recordings were performed for SR Ca^{2+} leak measurements.

CONCLUSION

In conclusion, severe hypoxia increased SR Ca^{2+} leakage and decreased *RyR2* gene expression. Treatment with JTV-519 reversed calcium leakage through direct interaction with RyR2 proteins but did not affect protein or gene expression. Additionally, JTV-519 increased gene expression of *RyR2* under normoxic but not hypoxic conditions. The presented data provide a molecular basis for JTV-519 to be considered as a potential pharmacological treatment option for OSA-associated heart failure. Future studies are needed to investigate the outcomes of JTV-519 in animal models and human patients with clinically significant OSA.

Ethics Committee Approval: This study was conducted on commercially available cellular line, no animals or humans were involved, thus ethical commitee approval is not applicable in this case.

Peer-review: Externally peer-reviewed.

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