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RESEARCH LETTER

Sildenafil normalizes MALAT1 level in diabetic cardiomyopathy

Lorenza Bacci[1] · Saviana A. Barbati[2] · Claudia Colussi[3] · Aurora Aiello[1,3] · Andrea M. Isidori[4] · Claudio Grassi[2,5] · Alfredo Pontecorvi[1,5] · Antonella Farsetti[3] · Carlo Gaetano[6] · Simona Nanni[1]

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Introduction

A large body of evidence recently highlighted the involvement of long non-coding RNAs (lncRNAs) in cardiovascular disease [1] and some dysregulated lncRNAs have been associated with diabetic cardiomyopathy [2–5]. Among them, a higher expression of the lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) has been observed in diabetic cardiomyopathy [6, 7]. However, a clear understanding of the molecular mechanisms leading to pathological regulation of lncRNAs in diabetic cardiomyopathy is still missing. Our prior work by Barbati et al. [8], established that, in the presence of high glucose, nitric oxide (NO) signaling derangement might alter the epigenetic landscape of cardiac cells, both in vitro and in vivo, via transcription factor CREM activation.

Aim

The present study is aimed at investigating the role of high glucose (HG) and NO pathway in the regulation of MALAT1 in the heart of mice after 6 months of prolonged hyperglycemia and in two cellular models of cardiomyocytes exposed to HG.

Methods

Cell lines, treatments, and RNA interference

Mouse HL-1 cells and rat H9C2 differentiated into cardiomyocytes were cultured and treated with high glucose (30 mM) for 72 h, DETANO and Sildenafil as in [8]. CREM silencing was performed with siRNA (TriFECTa and DsiDNA duplex, Integrated DNA Technologies) according manufacture's instruction as in [8].

Animal care and treatment

All experiments were performed in accordance with European Community guidelines and upon Approval of the Italian National Institute of Health (DGSAF0005330 n° 202/2016-PR) and ethical committee of Università Cattolica as described in [8]. Male CD1 mice were made hyperglycaemic by streptozotocin (STZ) injection and analyzed at 6 months after STZ treatment. Sildenafil treatment were as in [8].

RNA extraction and qRT

Analysis was performed as in [8] using the following primers designed on (NR_002847) and (XR_350899) sequences, respectively:

mMALAT1 5′-GTAGGTTAAGTTGACGGCCGTTA-3′ and 5′-ATCTTCCCTGTTTCCAACTCATG-3′;

rMALAT1 5′-CCTTTTTAATTACTTCAGTTGTAGCTTTGAC-3′ and 5′-TGATGGAGCCCAGCAGTTTAG-3′.

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✉ Carlo Gaetano
carlo.gaetano@icsmaugeri.it

✉ Simona Nanni
simona.nanni@unicatt.it

[1] Institute of Medical Pathology, Università Cattolica di Roma, 00168 Rome, Italy

[2] Institute of Human Physiology, Università Cattolica di Roma, 00168 Rome, Italy

[3] Institute of Cell Biology and Neurobiology, National Research Council, 00143 Rome, Italy

[4] Department of Experimental Medicine, "Sapienza" University, 00161 Rome, Italy

[5] Fondazione Policlinico Universitario Gemelli, 00168 Rome, Italy

[6] Laboratorio di Epigenetica, Istituti Clinici Scientifici Maugeri, via Maugeri 4, 27100 Pavia, Italy

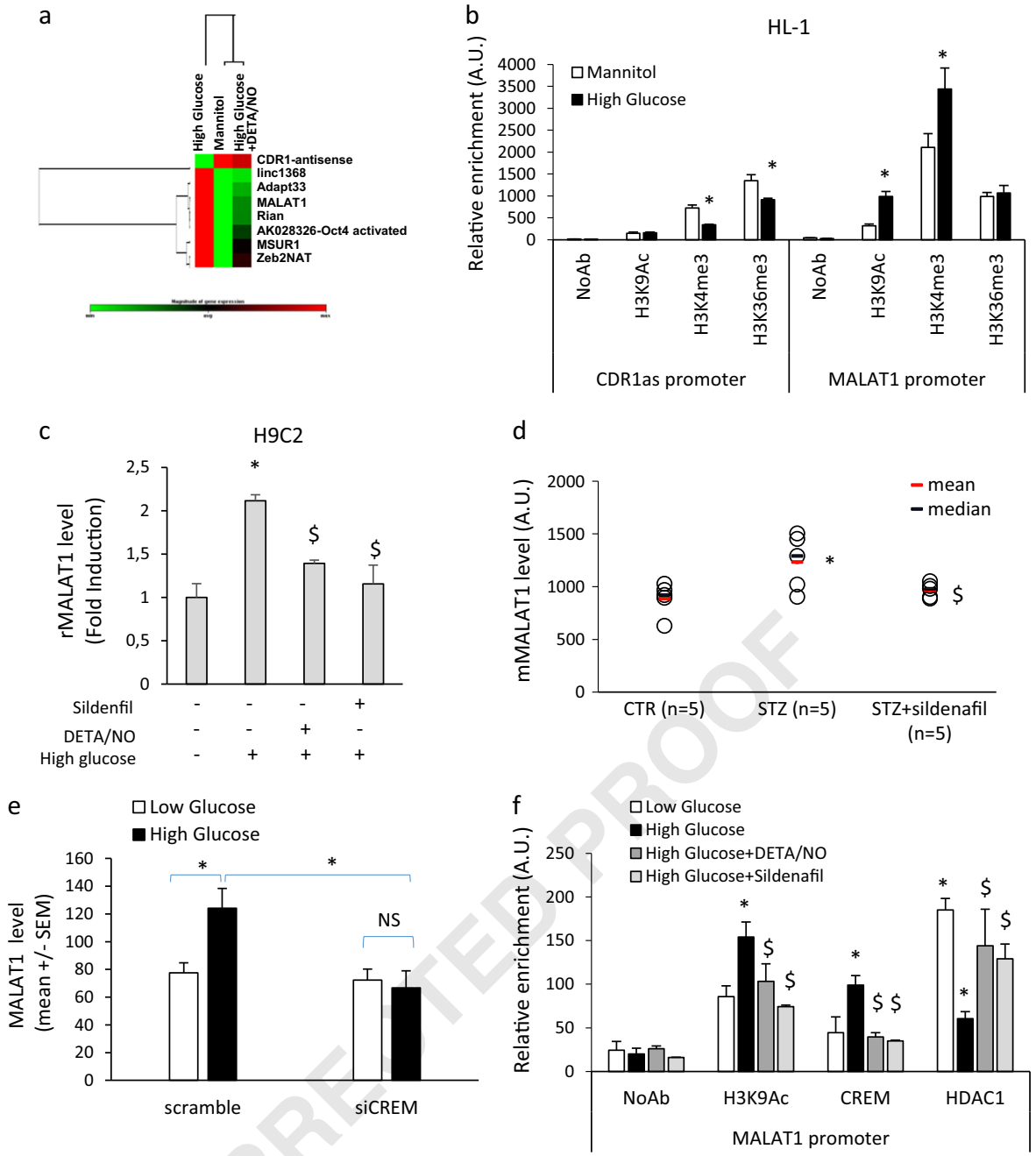

Fig. 1 Nitric oxide counteracts MALAT1 upregulation in cardiomyocytes exposed to high glucose and in heart of hyperglycemic mice. **a** Cluster analysis of down or up-regulated lncRNAs ($n = 8$, $p < 0.05$) in HL-1 cells treated with High Glucose (30 mM) in presence or absence of DETA/NO vs. Mannitol (30 mM) as control. **b** ChIP analysis of CDR1as and MALAT1 promoters in HL-1 cultured in high and low glucose performed with antibodies reacting to H3K9Ac, H3K4me3 or H3K36me3. No antibody sample (NoAb) was used as control. Data represent the mean ± SEM of three independent experiments. *$p < 0.05$ high glucose vs. low glucose. **c** MALAT1 expression was validated in differentiated H9C2 by qPCR. Data are expressed as fold induction and are mean ± SEM of four independent experiments. *$p < 0.05$ high glucose vs. control, $$p < 0.05$ high glucose plus DETA/NO or Sildenafil vs. high glucose. **d** MALAT1 levels analyzed by qRT-PCR in CD1/Saline (CTR), CD1/STZ mice treated with/without Sildenafil (STZ + Sildenafil) at the 3 months time-point after STZ injection. Sildenafil treatment was administered for 12 weeks. Sample size for each group is indicated. *$p < 0.05$ STZ vs. CTR; $$p < 0,05$ STZ + Sildenafil vs. STZ. **e** Evaluation of MALAT1 level in differentiated H9C2 cells transfected with siCREM or scramble in basal condition (low glucose) and after high glucose treatment (30 mM) of 72 h. Results are mean ± SEM of three independent experiments.*$p < 0.05$, NS = not significant. **f** ChIP analysis preformed on MALAT1 promoter with antibodies reactive to CREM, HDAC1, H3K9Ac. The NoAb condition was used as control. Experiments were performed in differentiated H9C2 treated with high glucose and cultured in presence or absence of DETA/NO or Sildenafil. Data are represented mean ± SEM of three independent experiments. *$p < 0.05$ high gluc vs. low gluc; $$p < 0.05$ high gluc + Sildenafil vs. high gluc

Gene expression by qPCR

Analysis was performed as in [9] using LncRNA Profiler qPCR Array Kit (SBI System Biosciences) according manufacturer's instruction.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as in [8] using the following primers:
mCDR1as promoter 5′-ATATGTCCACGGGTGTACA ATGAT-3′ and 5′-CGGTCTATGGATGAGGCTCTTG-3′;
mMALAT1 promoter 5′-CCTTTCCCCTCCGTCGT AGT-3′ and 5′-CCGTGGCGGCAAGGT-3′;
rMALAT1 promoter 5′-TGCGAAGGGACACGTCA CT-3′ and 5′-GGCCCACGCACCATCA-3′.

Statistical analysis

Data are expressed as mean ± SEM as indicated in figure legends. Statistical analyses were performed by using Sigma Plot 13. Significance was calculated using a two-tailed *t*-test or one-way ANOVA. Differences between groups were calculated with ANOVA and post-hoc Tukey HSD test with Bonferroni correction. A p-value of <0.05 was considered significant.

Results

Nitric oxide counteracts MALAT1 upregulation in cardiomyocytes exposed to high glucose and in heart of hyperglycemic mice

Mouse HL-1 and rat cardiomyocytes obtained from differentiated H9C2 cells were treated with high glucose for 72 h in the presence or absence of the NO donor, DETA/NO, or the PDE5 inhibitor Sildenafil. In addition, STZ injected CD1 mice, treated or not with Sildenafil, have been used as an in vivo model of cardiac damage determined by 6 months of prolonged hyperglycemia. First, we analyzed the expression of 90 lncRNAs in HL-1 cells by qPCR profiling. Among those, a subset of 30 lncRNAs was found expressed in HL-1 (Supplemental Fig. 1a). Eight of them ($p < 0.05$) were significantly down or up-regulated by HG condition compared to control (Fig. 1a). In this condition, treatment with DETA/NO normalized expression of the lncRNAs modulated by HG (Fig. 1a). To molecularly investigate the mechanism involved in this effect we performed a series of Chromatin immunoprecipitations (ChIPs) on lncRNAs up or down-regulated by HG including CDR1as and MALAT1 (Fig. 1b). Specifically, we studied the enrichment modulation of epigenetic marks on histone H3 such as tri-methyl lysine 4 (H3K4me3), tri-methyl lysine 36 (H3K36me3) and the lysine 9 acetylation (H3K9ac) [10]. In HG, H3K4me3 and H3K36me3 were significantly downregulated on CDR1as promoter whereas H3K4me3 and H3K9Ac were upregulated on that of MALAT1 suggesting that the modulation observed at RNA level (Fig. 1a) may rely on transcriptional regulation. To further confirm the key role of NO in the HG-dependent lncRNA alteration, we used a different cellular model the H9C2 cells differentiated into cardiomyocytes and exposed to HG in the presence/absence of DETA/NO or Sildenafil that directly elevated the intracellular level of NO and cGMP, respectively. As expected, MALAT1 upregulation induced by HG was significantly counteracted by the co-treatment with DETA/NO or Sildenafil (Fig. 1c). Of note, Sildenafil was effective in counteracting the negative consequences of hyperglycemia also in vivo. Indeed, the heart of STZ-injected mice showed a significant increase of MALAT1 in STZ-treated mice compared to controls (Fig. 1d). In this condition, Sildenafil administrated daily for 12 weeks, starting 3 months after STZ injection, rescued the effect of hyperglycemia reducing the level of MALAT1 (Fig. 1d). To gain further insights about the molecular mechanism controlling the expression of MALAT1 in the presence of HG, we analyzed the potential involvement in its transcription of transcriptional factor CREM previously identified as involved in gene expression determined by hyperglycemia [8]. Of interest, mouse and rat MALAT1 promoters have a similarly structured regulatory region at −700 bp from the transcriptional start site enriched in CREM binding sites as determined by transcription factor database (TRANSFAC 8.3) analysis. Remarkably, silencing of CREM in H9C2 cells completely abrogated the induction of MALAT1 mediated by HG treatment (Fig. 1e). ChIP analysis of MALAT1 promoter (Fig. 1f) revealed a significant CREM recruitment in the presence of HG. A condition which was inhibited by DETA/NO or Sildenafil. In parallel, the enrichment of H3K9Ac in the presence of HG was completely rescued by treatment with DETA/NO or Sildenafil. On the opposite, the recruitment of the Histone Deacetylase 1 (HDAC1), reduced by HG, was normalized in the presence of DETA/NO or Sildenafil. Accordingly, ChIP performed on cardiac chromatin, isolated 6 months after STZ treatment, showed a significant increase of CREM binding on MALAT1 promoter in STZ vs. saline-treated mice paralleled by an induction of H3K9ac level and a reduction of HDAC1 occupancy (Supplemental Fig. 1b) thus further confirming CREM as mediator of hyperglycemia-dependent MALAT1 upregulation in vivo.

Discussion

Taken altogether, these data suggest that Sildenafil counteracts the increase of MALAT1 occurring in cardiomyocytes as a consequence of elevated glucose levels in vitro

and in vivo. In light of the present data we reasoned that the following considerations might be extrapolated: (i) reduction of nitric oxide or cGMP bio-availability caused by prolonged hyperglycemia impairs lncRNA expression and (ii) Sildenafil, restoring function of nitric oxide signaling normalized MALAT1 expression levels. To our knowledge, this is the first observation that nitric oxide signaling is involved in the transcriptional regulation of MALAT1, a lncRNA possibly implicated in glucose sensing and diabetic cardiomyopathy.

Reduction of NO bio-availability is an early and key determinant in diabetic cardiomyopathy impairing both endothelial and cardiac function ([11] and references therein). The present study reveals that restoring the intracellular impact of NO signaling might be important to reverse the effect of hyperglicemia also controlling transcription of lncRNAs involved in cardiac injury and in the development of cardiomyopathy associated to dysmetabolic conditions including diabetes. Indeed, this mechanism might be part of a more complex process determining the cardioprotective effect of Sildenafil in heart failure [12, 13] as well as in diabetic cardiomyopathy [14, 15] suggesting novel therapeutics strategies in the presence of prolonged hyperglycemia.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institutional and/or national research committee.

Informed consent This article does not contain any studies with human participants performed by any of the authors.

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