Since STAT3 activation is involved in tumor progression and metastasis, we investigated the effect of GSNO in cell culture and mouse xenograft model of head and neck squamous cell carcinoma (HNSCC). GSNO treatment of HNSCCN cell lines reversibly decreases the activation (phosphorylation) of STAT3 in a concentration dependent manner. The reduced STAT3/NF-kB activity by GSNO correlated with decreased cell proliferation and increased apoptosis of HNSCC cells. In HNSCC mouse xenograft model, the tumor growth was reduced by systemic treatment with GSNO and was further reduced when the treatment combined with radiation and cisplatin. Accordingly, GSNO treatment also resulted in decreased levels of pSTAT3 and tumor growth regulators (ie. cyclin D2, VEGF and Bcl-2) in tumor tissue. In summary, these findings have implications for the development of new therapeutics targeting of STAT3 for treating diseases associated with inflammatory/immune responses and abnormal cell proliferation, including cancer.

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Mechanisms Of Hypoxia-Induced Immune Escape In Cancer And Their Regulation By Nitric Oxide

Charles Graham, Ivraym Barsoum, Judy Kim, Madison Black, Robert D. Siemens

Queen's University, Kingston, Canada

The acquired ability of tumour cells to avoid destruction by immune effector mechanisms (immune escape) is important for malignant progression. Also associated with malignant progression is tumour hypoxia, which induces aggressive phenotypes such as invasion, metastasis and drug resistance in cancer cells. Our studies revealed that hypoxia contributes to escape from innate immunity by increasing tumour cell expression of the metalloproteinase ADAM10 in a manner dependent on accumulation of the alpha subunit of the transcription factor hypoxia-inducible factor-1 (HIF-1α). Increased ADAM10 expression leads to shedding of the NK cell-activating ligand, MICA, from the surface of tumour cells, thereby resulting in resistance to NK cell-mediated lysis. Our more recent studies demonstrated that hypoxia, also via HIF-1α accumulation, increases the expression of the inhibitory co-stimulatory ligand PD-L1 on tumour cells. Elevated PD-L1 expression leads to escape from adaptive immunity via increased apoptosis of $CD8^+$ cytotoxic T lymphocytes. Accumulating evidence indicates that hypoxia-induced acquisition of malignant phenotypes, including immune escape, is in part due to impaired nitric oxide (NO)-mediated activation of cGMP signalling and that restoration of cGMP signalling prevents such hypoxic responses. We have shown that NO/cGMP signalling inhibits hypoxia-induced malignant phenotypes likely in part by interfering with HIF-1α accumulation via a mechanism involving calpain. These findings indicate that activation of NO/cGMP signalling may have useful applications in cancer therapy.

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Evaluating The Role Of Nitric Oxide Synthase In Oncogenic Ras-Driven Tumorigenesis

Chris Counter

Duke University Medical Center, Durham, USA

We previously reported that oncogenic KRAS activation of the PI3K/AKT pathway stimulates the remaining wild-type HRAS and NRAS proteins in a manner dependent upon both eNOS expression and C118 in HRAS and NRAS, which promoted tumor growth. Interestingly however, we recently found that loss of wild-type HRAS, NRAS, and even more potently, loss of both of these genes actually enhanced oncogenic KRAS-driven early tumorigenesis. Taken together, these results indicate that wild-type RAS proteins are tumor suppressing early in tumorigenesis, but tumor promoting in more malignant settings. Knock-in of a C118S mutation into an endogenous wild-type *RAS* gene did not, however, hamper oncogenic KRAS-driven tumor initiation. As such, redox-dependent reactions with C118 of wild-type RAS proteins are unlikely to be responsible for the tumor suppressive role of wild-type RAS proteins. This suggests that the redox-dependent reactions with C118 of wild-type RAS proteins are more important in more malignant settings. Given this, it stands to reason that inhibiting redox-dependent reactions like *S*-nitrosylation of wild-type RAS proteins may be more effective in established cancer settings. Indeed, we find that in three different models of KRAS-driven cancers-skin, pancreatic and lung- the general NOS inhibitor L-NAME reduced tumor burden and/or extended the lifespan of mice. Since oncogenic RAS has so far proven refractory to pharmacologic inhibition, targeting NOS activity may be an actionable approach to inhibiting RAS signaling for the treatment of a broad spectrum of cancers.

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Young Investigation Session Selected Oral Communications

Nitric Oxide And Hypoxia Response In Pluripotent Stem Cells

Estefanía Caballano Infantes, Ana Belén Hitos Prados, Irene Díaz Contreras, Gladys M. Cahuana, Abdelkrim Hmadcha, Franz Martín Bermudo, Bernat Soria, Juan R. Tejedo Huamán, Francisco J. Bedoya Bergua

Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Sevilla, Spain

The expansion of pluripotent cells (ESCs and iPSCs) under conditions that maintain their pluripotency is necessary to implement a cell therapy program. Previously, we have described that low nitric oxide (NO) donor diethylenetriamine/nitric oxide adduct (DETA-NO) added to the culture medium, promote the expansion of these cell types. The molecular mechanisms are not yet known. We present evidences that ESC and iPSCs in normoxia in presence of low NO triggers a similar response to hypoxia, thus maintaining the pluripotency. We have studied the stability of HIF-1α (Hypoxia Inducible Factor) in presence of low NO. Because of the close relationship between hypoxia, metabolism, mitochondrial function and pluripotency we have analyzed by q RT-PCR the expression of genes involved in the glucose metabolism such as: HK2, LDHA and PDK1; besides other HIF-1α target gene. We further analyzed the expression of genes involved in mitochondrial biogenesis such as PGC1α, TFAM and NRF1 and we have

observed that low NO maintains the same pattern of expression that in hypoxia. The study of the mitochondrial membrane potential using Mito-Tracker dye showed that NO decrease the mitochondrial function. We will analyze other metabolic parameters, to determinate if low NO regulates mitochondrial function and mimics Hypoxia Response. The knowledge of the role of NO in the Hypoxia Response and the mechanism that helps to maintain self-renewal in pluripotent cells in normoxia, can help to the design of culture media where NO could be optimal for stem cell expansion in the performance of future cell therapies.

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Redox Regulation Of Metabolic And Signaling Pathways By Thioredoxin And Glutaredoxin In Nitric Oxide Treated Hepatoblastoma Cells

C. Alicia Padilla Peña, Raúl González, María J. López-Grueso, J. Antonio Bárcena

Department of Biochemistry and Molecular Biology, IMIBIC, University of Córdoba, Córdoba, Spain

Background: NO has an antiproliferative action on HepG2 cells and Thioredoxin (Trx) and Glutaredoxin (Grx) have denitrosilase and deglutathionylase activities.
Aims: To ascertain whether Trx and/or Grx systems intermediate the anti-proliferative effect of NO on hepatoblastoma cells by modulating the redox-state of key proteins.
Methods: HepG2 cells overexpressing Nitric Oxide Synthase-3 (NOS-3) were transfected with specific siRNA to silence Trx1 and Grx1. The expression and thiolic redox state of proteins were determined by Western blot and redox mobility shift assay.
Results: Overexpression of NOS3 increased the levels and activities of proteins of the redoxin systems, Trx1, Grx1, TrxR1 and TxnIP, and the levels of signaling proteins (Akt1, pAkt1^{-}Ser473, MapK, pMapK, Stat3, Fas). The thiolic redox state of Trx1, Grx1 and Akt1 shifted to more oxidized. Increases were also observed in Pro-apoptotic Caspase-3 fragment levels; caspase 3, 8 and 9 activities; antiapoptotic (Bcl-2); mitochondrial energetic (Aco2) and heme (Urod) metabolism; Glycolysis (Pkm2); and pentose phosphate pathway (Tkt). However, two cytosolic proteins related to iron (Aco1) and one carbon (Mat2) metabolism decreased markedly. Moreover, the redox state of Urod and Aco1 shifted to more oxidized cysteines.

Trx1 or Grx1 silencing augmented Tyr nitration and diminished cell proliferation in WT cells, but attenuated the antiproliferative effect on NO, the increase of Fas, Akt1 and pAkt1^{-}Ser473 and the oxidative modification of Akt1 in NOS3 cells.
Conclusions: Trx1 and Grx1 exert contradictory influences on HepG2 cells. They are required for proliferation but they also contribute to antiproliferative effect of NO, associated to Akt1 redox changes.

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Session 4: Regulation of Immune Response by Nitric Oxide
Moderator: Dr. Khosrow Kashfi
INVITED SPEAKERS

Cellular Protective Mechanisms Of Inducible Nitric Oxide Synthase

Timothy R. Billiar

Department of Surgery, University of Pittsburgh, USA

The inducible nitric oxide synthase (iNOS) is expressed constitutively but also induced in a number of epithelial cell types. iNOS regulates a number of cellular processes in these cell types without exerting toxicity. Among these functions is protection from cellular injury mediated by pro-apoptotic signals. We have had long-standing interest in the cell protective roles of iNOS in hepatocytes. We demonstrated that the upregulation of iNOS protects hepatocytes and the liver from TNF-mediated toxicity. This includes the inhibition of caspase activity through s-nitrosation. However, some of the effects are mediated through cGMP. Exploration into the mechanisms of the cGMP-mediated protection identified a role for the iNOS/NO/cGMP pathway in the activation of ADAM17 (TACE), which is a sheddase that cleaves a number of cell surface receptors including TNF receptor type 1 (TNFR1). The activation is associated with the phosphorylation of TACE.

The iNOS/NO/cGMP/TACE pathway can be augmented by PDE5 inhibitors and reduce organ injury in the setting of sepsis. The implications go beyond acute pathophysiology and may be important to the mechanisms of iNOS in promoting aggressive cancers.

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Post-Translational Nitric Oxide–Dependent Modifications In Immune System

Antonio Martínez-Ruiz

Hospital Universitario de la Princesa, Instituto de Investigación Sanitaria Princesa (IP) Madrid, Spain

Nitric oxide non classical signalling is exerted through a series of covalent protein post-translational modifications, which include modification of cysteine residues by S-nitrosylation and S-glutathionylation.

A key process in adaptive immunity is the immune synapse that tightly couples T cells with antigen presenting cells, triggering antigen recognition by T cells. In this highly regulated process, we have shown that eNOS is activated, inducing protein S-nitrosylation. While both N-Ras and K-Ras are present in T cells, only N-Ras, which colocalizes in the Golgi with eNOS, is S-nitrosylated and activated during the immune synapse, providing an example of short-range selectivity of NO signalling through S-nitrosylation.

We have developed proteomic methods to detect S-nitrosylation and reversible cysteine oxidations. We have applied them to detecting S-nitrosylated proteins in macrophage activation, highlighting the role of denitrosylase mechanism, particularly the thioredoxin pathway, in protecting macrophages from self-modification.