Increased oxidative stress is associated with chronic intermittent hypoxia-mediated brain cortical neuronal cell apoptosis in a mouse model of sleep apnea

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Obstructive sleep apnea (OSA)

OSA: significant and highly prevalent public health problem that leads to substantial
• neurobehavioral and cardiovascular morbidities i.e.
  • hypertension,
  • myocardial infarction (MI),
  • stroke

(Young et al., 1996, 2002)

Recent studies indicated that patients with OSA display
• increased systemic markers of oxidative stress (Carpagnano et al., 2002; Montplaisir et al., 1992)
  • Inflammatory marker, i.e. IL-6
  • Oxidative stress marker, i.e. 8-isoprostane
• Loss of gray matter in cognitively relevant brain regions (cortico-hippocampal region) (Macey et al., 2002, 2003)

Chronic intermittent hypoxia (CIH)

• Chronic intermittent hypoxia (CIH), as occur in OSA, is associated with substantial cortico-hippocampal damage, leading to impairment of neurocognitive, respiratory and cardiovascular function
• Previous study in a rat model have shown that CIH increases brain cortical neuronal cell death
• However, the molecular events leading to CIH-mediated neuronal death remain large undefined.
• The oscillation of $[O_2]$ during CIH remarkably mimics the processes of ischemic-reoxygenation could increase reactive oxygen species (ROS)

Aim of study

• To establish a mouse model of OSA
• to identify molecular mechanisms underlying cortical neuronal cell death

Ischemic-reperfusion (IR) injury

Xanthine oxidase (XO)
Irreversible
Xanthine dehydrogenase (XD)

Inosine $\rightarrow$ Adenosine $\rightarrow$ AMP $\rightarrow$ ADP $\rightarrow$ ATP breakdown

Hypoxanthine and xanthine

Fenton reaction

O$_2^-$ ARG
SOD
\nonumber

$\text{OH}^-$ $\text{NO}^\cdot$ $\text{H}_2\text{O}_2$

$\text{SOD}$ $\text{NOS}$ $\text{NO}$ $\text{SNOD}$
Methods to determine

- Behavioral deficit
- Detect ROS production in mouse brain cortical neurons
- Increase expression of oxidative stress response markers
  - c-Fos, c-Jun
  - NF-kB
- Caspase-3 activation
- Result from long-term exposure to CIH
- Apoptosis cell

Morris water maze

Fluorescent oxidation assays
  - Dichlorofluorescein (DCF)
  - Hydroethidine (HEt)

Immunohistochemical staining

Protein oxidation assay
  - Lipid peroxidation assay
  - Nucleic acid oxidation assay

Animals

- C57BL/6J mice (black 6)
- Transgenic mice overexpressing SOD1

MATERIAL & METHOD

- Normoxic control mice (NOX) were placed in chambers but were not exposed to hypoxic gas

RESULTS

- Chronic intermittent hypoxia conditions
  - Duration of CIH exposure 1, 3, 5, 7, 14, 21 and 30 days

Statistical analysis

- Results were expressed as mean ± S.E.
- Student t-test and ANOVA
- Student-Newman-Keuls’ post hoc tests were used when significant differences
- P-value less than 0.05 was considered statistically significant
Methods to determine

<table>
<thead>
<tr>
<th>Behavioral deficit</th>
<th>Morris water maze</th>
</tr>
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</table>
| Decrease ROS production in mouse brain cortical neurons | Fluorescence oxidation assay
| Increase expression of oxidative stress response markers | NADPH oxidase assay
| Result from long-term exposure to CIH | Protein oxidation assay
| Caspase 3 activation | Lipid peroxidation assay
| Apoptosis cell | Nucleic acid oxidation assay

Increase expression of oxidative stress response markers

- C-Fos, c-Jun
- C-Fos, c-Jun
- Caspase 3 activation

**Effect of CIH on spatial reference learning and memory and spatial working memory in mice**

**Morris water maze**

Animals were kept on a 12-h light/dark schedule

(6:00 a.m.–6:00 p.m.)

All behavioral testing was tested during the light phase

**Spatial reference task acquisition**

**Spatial reference learning and memory**

- 3 trials/session
- Inter-trial interval = 10 min
- 90 sec to escape platform and 15 sec to stay

- Probe trial: 24h later

**Working spatial memory**

- 4 trial/session
- 1st trial: information trial
- 2nd-4th trial = retention trial
- Time between 1st, 2nd trial = 120 min
- Time between 2nd, 3rd, 4th = 30 sec
Results

CIH conditions and water maze performance in mice: place training

Results

CIH conditions and water maze performance in mice: probe trial

Methods to determine

Assay of ROS production in mouse brain cortex and cortical neuronal cell

Assays of ROS production by Dicholorfluorescein (DCF) and hydroethidine (HEt) oxidation

0.2 ml saline buffer containing 20% of DMSO alone (vehicle control)
Results

ROS production in mouse brain cortical neuronal cells: C57BL/6J mouse (control)

HEt  DCF
NOX  CIH

Results

ROS production in control and SOD1-transgenic mice

HEt oxidation  DCF oxidation

These data together indicated that CIH induced ROS production in mouse brain cortex and in brain cortical neuronal cells

Methods to determine

- Behavioral deficit
- Morris water maze
- Detect ROS production in mouse brain cortical neurons
- Fluorescent oxidation assay
- Hydroethidine (HEt)
- Increase expression of oxidative stress response markers
  - c-Fos, c-Jun
  - NF-κB
- Immunohistochemical staining
- Immunohistochemical staining
- Caspase-3 activation
- Lactate dehydrogenase (LDH)
- Apoptosis cell
- Immunohistochemical staining
- Protein oxidation assay
- Lipid peroxidation assay
- Nucleic acid oxidation assay

Effect of CIH on oxidative stress response gene expression

- Immunohistochemical staining
  - c-Fos expression
  - c-Jun expression
- LacZ (β-Gal) reporter gene activity assay
  - NF-κB expression
Immunohistochemical staining

- Anaesthetized
- Perfused
- 10 ml 0.9% saline buffer
- 10 ml 4% PFA
- Dissected
- Blocked
- Washed
- 0.2% Triton X-100 in PBS 5 min six times
- Incubated
- Specific antibody overnight at 4°C

Results

1-Fos expression in mouse brain cortex

<table>
<thead>
<tr>
<th>NOX</th>
<th>CIH1</th>
<th>CIH2</th>
<th>CIH3</th>
</tr>
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<tbody>
<tr>
<td><img src="CIH7.png" alt="Image" /></td>
<td><img src="CIH14.png" alt="Image" /></td>
<td><img src="CIH21.png" alt="Image" /></td>
<td><img src="CIH30.png" alt="Image" /></td>
</tr>
</tbody>
</table>

NF-kB expression by LacZ (β-Gal) reporter gene activity assay

**For wholemount staining**

- Anaesthetized
- Perfused
- 10 ml 0.9% saline buffer
- 10 ml 4% PFA
- Dissected
- Postfixed
- Washed
- Incubated
- Washed
- 2% PFA at 4°C 30 min

Leica microscope attached to a video camera system

Results

NF-kB transcriptional activation in mouse brain cortex

<table>
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<th>CIH1</th>
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**For histological staining**

- Cut
- Collected
- Superfrost Plus slides
- Stained as in wholemount staining

Results

NF-kB transcriptional activation in mouse brain cortex

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NF-κB expression by LacZ (β-Gal) reporter gene activity assay

**For activity assay**

- Anaesthetized
- Pentobarbital (50 mg/kg)
- Perfused
- 20 ml 0.9% saline buffer
- Sonicated
- 0.9% saline buffer with protease inhibitors

Protein concentration determination and used commercial β-gal activity assay

- NF-κB expression by LacZ (β-Gal) reporter gene activity assay

**Methods to determine**

- Behavioral deficit
- Morris water maze
- Detect ROS production in mouse brain cortical neurons
- Fluorescent oxidation assay
- DCF
- Hydroethidine (HEt)
- C-Fos, c-Jun
- Immunohistochemical staining
- Sonicated
- Lipid peroxidation assay
- Nucleic acid oxidation assay
- CASP3 activation
- Immunohistochemical staining
- Apoptosis cell
- Immunohistochemical staining

**Results**

- Reactive oxygen species (ROS)
  - Oxidized
  - Protein Oxidation
  - Lipid peroxidation
  - DNA
  - Nucleic acid oxidation
  - Carboxyl compound
  - Malondialdehyde (MDA)
  - 8-hydroxyguanosine (8-OHG)

**Protein oxidation assay**

- Anaesthetized
- Pentobarbital (50 mg/kg)
- Perfused
- 20 ml 0.9% saline buffer
- Dissected
- 0.9% saline buffer
- 20 mM phosphate buffer (pH 6.5)
- Ethanol/ethyl acetate (1:1)
- 6 M guanidine HCl in 20 mM sodium phosphate buffer (pH 6.5)

The carbonylated residues in oxidized proteins can be derivatized with 2,4-dinitrophenylhydrazine (DNPH) and analyzed by spectrophotometry.

**Results**

- Protein oxidation product: carbonyl protein

- Fold increase in protein oxidation

- NOX
- C5H1
- C5H2
- C5H3
- C5H4
- C5H5
- C5H6
- C5H7
- C5H8
Lipid peroxidation assay

- Anaesthetised
- Perfused
- Dissected out
- Frozen in liquid nitrogen

20 mM phosphate buffer (pH 7.4) containing 0.5 M butylated hydroxytoluene

Lipid peroxidation was measured by malondialdehyde (MDA) production using a commercially available kit.

Results

Lipid peroxidation product: MDA

Nucleic acid oxidation assay

- anti-8-hydroxyguanosine antibody
- 3,3-diaminobenzidine staining

Methods to determine

- Behavioral deficit
- Morris water maze
- Detect ROS production in mouse brain cortical neurons
  by Fluorescent oxidation assays
- Increase expression of oxidative stress response markers
  - ER Stress
  - NF-κB
- Caspase-3 activation
- Apoptosis cell death

Assay of caspase-3 activation in mouse brain cortex and brain cortical neuronal cells

- Immunohistochemical staining
- Neuronal nuclear antigen (NeuN)
- Caspase-3
Immunohistochemical staining

- Anaesthetized rat
- Perfused
- 10 ml 0.9% saline buffer
- 10 ml 4% PFA
- Dissected out
- Embalmed
- Incubated 20% sucrose 12–16 h
- OCT
- Fixed
- Embedded
- Specific antibody overnight at 4 °C
- Triton X-100 in PBS 5 min six times
- Specific Ab conjugate with FITC or rhodamine 2 h at room temperature in the dark
- Mounted

Results

CIH conditions and caspase-3 activation in mouse brain cortex

- NOX
- CIH3
- CIH7
- CIH14
- CIH21
- CIH30

Methods to determine

- Behavioral deficit: Morris water maze
- Detect ROS production in mouse brain cortical neurons by Fluorescent oxidation assays, Dichlorofluorescein (DCF), Ethidium homodimer (EHD)
- Increase expression of oxidative stress response markers: NF-kB, c-Fos, c-Jun
- Caspase-3 activation: Immunohistochemical staining
- Protein oxidation assay
- Lipid peroxidation assay
- Nucleic acid oxidation assay

Analysis of apoptosis in mouse brain cortex and brain cortical neuronal cells

- TUNEL: TdT-mediated dUTP-biotin nick end labeling assay
- Immunohistochemical staining of sections with anti-single stranded DNA antibody (SS-DNA)

Results

CIH conditions and apoptotic cell death

- NOX
- CIH21
- CIH30
- NOX/SOD1
- CIH21/SOD
- CIH30/SOD
**Results**

**CIH conditions (21 days exposed) and apoptotic cell death**

- **NeuN**
- **SS-DNA**
- **Overlay**

**NeuN**

**TUNEL**

**Overlay**

17/10/2007

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**Results**

**The percentage of cortical neuronal cells apoptosis**

- **NOX**
- **CIH1**

- **TUNEL**
- **SS-DNA**

Percentage of neuronal cell apoptosis (%)

17/10/2007

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**DISCUSSION**

Increased cortical neuronal cell apoptosis may contribute to CIH-mediated neurocognitive dysfunction.

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**Discussion**

- Substantial evidences suggested that chronic intermittent hypoxia (CIH)
  - plays a major role in the pathophysiology of neurocognitive dysfunction of OSA.

- However, adequate animal models to reproduce the clinical and pathological features of OSA
  - not well established.

- Furthermore, the molecular events of CIH-mediated neuronal cell loss
  - remain unclear

17/10/2007

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**Previous studies in rats showed that,**

- Brain cortical neuronal cell damage by CIH because these cell populations are more vulnerable to hypoxia/intermittent hypoxia than neurons from other regions.
  - (Kring et al., 2002, 2003)

- Furthermore, OSA is associated with reduction of blood flow from the middle cerebral artery: potentially target to cortical neuronal cells.
  - (Knoue et al., 2001; Binder and Gozal, 2002; Hynksen et al., 1996;Bailers and Franklin, 1996)

- In addition, MRI studies:
  - substantial gray matter losses in selected brain cortical regions in OSA patients > normal controls.
  - (Macey et al., 2002, 2003)
Present study extended previous study in rats model to mouse models

- Define the optimal intermittent hypoxia conditions to mimic the neural consequence of OSA

- Use mouse model to study the molecular events contributing to CIH-mediated brain cortical neuronal vulnerability

May assist in our understanding of OSA-mediated neurocognitive dysfunction

Rat versus mouse CIH models

The magnitude of hypoxia to induce cortical neuronal apoptosis in C57BL/6 mice > that used in rats. (Gozal et al., 2001)

The mechanism underlying the decrease hypoxia susceptibility in mice is unclear

- levels of hypoxia used in the present study was similar to that used in arousal mechanisms in mice. (Tagaito et al., 2001)

In contrast, Sprague–Dawley rats, the level of hypoxia required to elicit arousal from either non-rapid eye movement (REM) or REM sleep > 9.3%. (D. Gozal, unpublished observations)

Thus, difference in intrinsic hypoxic chemoreceptor sensitivity and/or in the arousal mechanism seem to play a role in the magnitude of the hypoxia oscillations that occur in these two species when arousal is dependent on hypoxic challenge.

CIH-mediated cellular ROS production and molecular oxidation

To test the hypothesis that oscillation of [O₂] during CIH, like that of ischemic/reperfusion, may increase ROS production (SOD-1 transgenic mice)

Superoxide

General ROS production

HeT

DCP

CIH > NOX

SOD 1 transgenic mice

After CIH

oxidative stress response marker (gene expression)

c-Fos
c-JunNF-kB

Degradation of protein, lipid and nucleic acid

Support that enhanced ROS production may be an important causative factor associated with brain cortical neuronal damage incurred by CIH

Exposures to episodic hypoxia led to alterations in respiratory control (dorsal brainstem neurons), at least in part, by CIH induced changes in ROS production, because administration of SOD mimetics abolished such respiratory adaptations. (Peng and Prabhakar, 2003)

Taken together, it is plausible that their experimental findings support the paradigm of CIH-induced cortical neuronal cell death and neurological dysfunction are, at least partially by elevation of ROS production.

CIH that occurred from ischemia-reperfusion

ROS production

Cellular degradation and death

(Langer et al., 2003; Lavie 2003; Gonzalez et al., 2002)
Discussion

To test the hypothesis that:

- CIH ➔ ROS ➔ Caspase 3 activation ➔ apoptosis

(SOD-1 transgenic mice)

Percentage of neuronal cell apoptosis (%)

Caspase-3

CIH > NOX

SOD1 transgenic mice

Conclusion

Chronic intermittent hypoxia (CIH) as occur in OSA, activates oxidative stress response marker (gene expression).

- Caspase-3 activation
- Cell degradation and apoptosis
- Neurocognitive deficit

CIH that occurred from ischemia-reperfusion

ROS production

Cellular degradation and death

(Lozina et al., 2003; Lavie 2003; Gonzalez et al., 2002)
Nitric Oxide (NOS) → Calcium
  → Mitochondria → Cytochrome C → Caspase → DNA Fragmentation → Apoptosis
  → Energy Failure → Necrosis → Nuclear/Cytoplasmic Breakdown
Factors that induce NFκB

Cytokines (e.g., TNFα, IL-1, IL-6, IL-12, IL-18)
Infection (bacterial toxins, e.g., HSP60)
Apoptosis Inducers (anticancer agents, cytokines)
Endotoxin (LPS)
Carcinogens
Stress (life, hypoxia, heavy metals)

Potential Mechanisms of Injury Following Hypoxia-Ischemia

HYPOXIA-ISCHEMIA

ANAEROBIC GLYCOGENESIS

ADENOSINE
GLUTAMATE
LACTATE
HYPOXANTHINE
NMDA RECEPTOR
INTRACELLULAR XANTHINE OXIDASE
ACTIVATES LIPASES
ACTIVATE NOS
NITRIC OXIDE
FREE FATTY ACIDS
OXIDATION

Leukemia 16:1053-1068, 2002
**Control of Respiration DRG**

- The dorsal respiratory group (DRG) is responsible for normal quiet inspiration.
- At usual blood gas levels, DRG generates action potentials spontaneously about 12 times per minute.
- The signals begin weakly and ramp up over two seconds and then stop abruptly for about 3 seconds.
- The output from these is mainly to the diaphragm and is responsible for inspiration during quiet breathing.
- The DRG can be considered the main respiratory pacemaker at rest.

**Basic rhythmic breathing and Inspiratory Neuronal activity**

The basis of rhythmic breathing. During inspiration the activity of inspiratory neurons increases steadily (ramps up). At the end of inspiration, the activity shuts off abruptly and expiration occurs by virtue of elastic recoil of lungs.

**Potential Mechanisms of Injury Following Hypoxia-Ischemia**

- Anaerobic Glycolysis
- Adenosine
- Hypoxanthine
- Xanthine oxidase
- Activates lipases
- Activates NOS
- Nitric oxide
- Oxygen
- Free radicals

**Potential Strategies for Preventing Reperfusion Injury**

- Mild hypothermia
- NMDA receptor blockers
- Superoxide dismutase
- Nitric oxide synthase inhibitors
- Free radicals
HYPOTHESIS:

**Anaerobic Glycolysis**

- ATP
- Glucose

**NMDA Receptor**

- Calcium influx
- Cytochrome C
- Apaf-1
- Pro-caspase 9
- Apoptosome
- active caspase 9
- Apoptosis

**BCL-2 and BAX proteins regulate mitochondrial permeability**

- BAX
- BCL-2

**BCL2/BAX balance serves as a kind of dimmer**

David Vaux in 1988 suggest that increased levels of bcl-2 promotes cell survival by inhibiting apoptosis.