Hydrogen peroxide:
a clue to understand the high frequency of thyroid nodules

32th Meeting
12th April 2008

Natacha Driessens
Hydrogen peroxide ($H_2O_2$) in thyroid: biosynthesis of thyroid hormones

Apical Pole
- $I^-$ channel
- $Na^+/K^+$ ATPase
- $NIS$
- TPO
- $H_2O_2$
- $H_2O + 1/2 O_2$
- $TgI$
- $Tg$
- $H_2O_2$-generating System (DUOX 1/2)

Basal Pole
- $I^-$ channel
- $Na^+$
- $K^+$
- $Na^+/K^+$ ATPase
- $T4 + T3$
- $TSH$
- $T4 + T3$
- $Tg$
- $O_2$
- $NADP^+ + H^+$
- $NADPH$
Thyroid oxidases: DUOX1 and DUOX2

• DUOX1 and DUOX2 cloned by sequence homology with NOX2 (gp91phox), catalytic subunit of H₂O₂-generating system of leukocyte (De Decken X et al. 2000)

Fig. 1. Structure and membrane topology of Nox family NADPH oxidases. All Nox/Duox enzymes contain six membrane-spanning domains, two homologous conserved motifs involved in NADPH and FAD binding. In addition to these structural features, Nox5 contains four calcium-binding EF-hand motifs in its N-terminus, whereas Duox proteins contain an additional transmembrane domain, a peroxidase-like domain and two EF-hand motifs.

Geiszt 2006
Thyroid oxidases: DUOX1 and DUOX2

- Ca\(^{2+}\)-dependent NADPH Oxidases (Dupuy C et al. 2005)

- Apical membrane localization of DUOXs proteins of thyrocyte

DUOX expression in WT adult mouse thyroid

Milenkovic et al 2006
Highly expressed in thyroid, however also expressed in trachea and lung (DUOX1) and in salivary glands and digestive tract (DUOX2)

2 glycosylated forms of protein (180 and 190 kDa)
190 kDa form expressed at apical membrane and functionally active (De Deken X, Wang D et al. 2002)

2 maturation factors DUOXA1 and DUOXA2 for respective DUOXs
> ER-to-Golgi transition, maturation (glycosylation) and translocation to apical membrane of functional DUOXs (Grasberger 2006)
DUOXs in human

- DUOX1 and DUOX2 in thyroid
- DUOX2 mutations related to permanent and severe congenital hypothyroidism through absence of all functional domains of the protein

1) Inactivating Mutations in the Gene for Thyroid Oxidase 2 (THOX2) and Congenital Hypothyroidism
   Moreno et al, NEJM 2002

2) Persistent Mild Hypothyroidism Associated With Novel Sequence Variants of the DUOX2 Gene in Two Siblings
   Vigone et al, HUMAN MUTATION 2005

3) Congenital hypothyroidism caused by new mutations in the thyroid oxidase 2 (THOX2) gene
   Pfarr et al, Clinical Endocrinology 2006

4) Three Mutations (p.Q36H, p.G418fsX482, and g.IVS19-2AC) in the Duox2 Gene Responsible for Congenital Goiter and Iodide Organification Defect
   Varela et al, Clinical Endo 2006
1) Biallelic Inactivation of the Dual Oxidase Maturation Factor 2 (DUOX2) Gene as a Novel Cause of Congenital Hypothyroidism

Grasberger et al. JCEM 2008

DUOXs in human

- DUOXA2 mutation related to congenital hypothyroidism

DUOX & DUOXA, by generating H$_2$O$_2$, are implicated in thyroid hormone biosynthesis
What are the arguments to think that H$_2$O$_2$ could be involved in the etiopathogenesis of thyroid nodules?
Epidemiology

- Clinically palpable thyroid nodules: 4–8 % > 40 years old
- Echographic visible nodules: 40–50 % > 60 years old
- 5 % thyroid cancers (80% papillary carcinoma)
- Annual incidence of thyroid cancer: \[
\begin{align*}
&\frac{1.2-2.6}{100,000} \text{ men} \\
&\frac{2.0-3.8}{100,000} \text{ women}
\end{align*}
\]
- Papillary microcarcinoma (tumour < 1 cm Ø): 0.3–13 % series of autopsy
Etiopathogenesis

- Iodide deficiency
  - prevalence of hot nodules
  - multi-nodular goiter
  - incidence of thyroid cancer
  - follicular C+ / papillary C+

- Irradiation = only environmental risk factor

- Papillary cancer < activation RAS/RAF/MAP Kinase
  - direct : mutation BRAF/RAS
  - indirect : activation Tyrosine Kinase R
  - < rearrangements RET/PTC
Hypothesis

* High frequency of thyroid tumours
* Polyclonal origin of multifocal papillary carcinoma

\[ \text{ROS} \]

\[ \text{H}_2\text{O}_2 \text{ production} \succ \text{hormones} \]

\[ \text{Long lifetime of thyrocyte} \]

\[ \text{H}_2\text{O}_2 \]

direct mutagenic effect

Oxidation of bases \[ \rightarrow \] errors of matching

DNA breaks
  - single-strand
  - double-strand
Protective mechanisms of thyroid cell against H$_2$O$_2$

**PHYSICAL**
- restricted apical localization of H$_2$O$_2$ production

**CHEMICAL**
- detoxifying enzymes
1. Physical

1. Compartmentation of DUOX with TPO: producer and consumer of H$_2$O$_2$

2. TPO and presumably DUOX stored inactive in granules just below apical membrane and their exocytosis is tightly regulated when necessary

3. Tight control of DUOX export from the ER to the membrane: role of DUOXA on delivering fully glycosylated and active DUOX at the cell membrane

4. Positive control of activity by iodide restricts H$_2$O$_2$ generation when iodide not available. (Corvilain)

Song et al JCEM 2007
2. DETOXIFICATION MECHANISMS

1) GSH peroxidases (Se) GSH reductase and NADPH2

2) Peroxiredoxin

3) Thioredoxin and thioredoxin reductases (Se)

4) Catalase (high Km)

Song et al. JCEM 2007
What happens when protective mechanisms are exceeded?

Fenton’s reaction:

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH}^- \]

DNA Damage

Kinases

ASK

Apoptosis

Repair

Mutations

Effects

Proliferation

Prot Y

Prot Ypase

Prot Yp

Prot Yp3

GFR

GF

Song et al. JCEM 2007
Possible mechanism of nodule appearance in case of iodine deficiency

EVIDENCE OF IN VIVO MUTAGENIC EFFECTS OF H$_2$O$_2$ ON THYROID

1) **In rat** (Maier et al, Endocrinology 2006)
   - Spontaneous mutation frequency is increased
     ~ 10x higher in thyroid than in other organs
   - Somatic mutations of TSH receptor:
     higher frequency of those resulting from DNA oxidation
   - More oxidized purines and pyrimidines in thyroid than in lung and liver measured by specific enzymes in Comet assay

2) **In human** (Detours et al, Br J Cancer 2007)
   - Discrimination of Chernobyl (radiation damage signature) and sporadic (H$_2$O$_2$ damage signature) papillary carcinoma on the basis of gene expression
EVIDENCE OF EXPERIMENTAL MUTAGENIC EFFECTS OF H₂O₂ ON THYROID
Experimental thyroid model for $\text{H}_2\text{O}_2$-induced DNA damage evaluation

* In vitro:  
  - rat (PCCl3)
  - human (HTori-3)
  - pig thyroid slices
  - human thyroid primary cultures

* Oxidative stress modulation $\rightarrow$ bolus of different [$\text{H}_2\text{O}_2$]

* Evaluation of DNA damage: $\text{H}_2\text{O}_2$ vs irradiation
Mutagenesis study

Direct carcinogen

Indirect carcinogen

metabolism

Gene mutations
- Ames test
- HPRT locus
- Mouse lymphoma assay
- TK loci

Interactions with DNA

DNA primary damage

DNA lesions

DNA repair

MUTATION

Malignant cell (cell transformation)

Clone of transformed cells

Comet assay

UDS

Sister chromatid Exchange

γH2AX

Back to normal

Chromosome aberration test

Micronuclei test

Chromosome damage
Comet assay (Single-Cell Gel Electrophoresis)

Single Cell Suspension

Preparation of Slides

Stain Slide

Cells, tissue, Cell line ...

Single & Double Strand Break Detection
DNA damage in rat thyroid cell line (PCCl3)

Dose-response by age at exposure

Age at exposure: <15

Age at exposure: ≥15

Rel. Risk

Dose (Gy)
$H_2O_2$ provokes DNA damage

- non lethal $[H_2O_2]$ (0.05mM to 0.5mM) provoke large number of strand breaks and as many as IR in different, *in vitro*, thyroid models

- potential carcinogen
Effect of depletion of glutathione on DNA damage induced by H$_2$O$_2$ on PCCl$_3$ cells

BSO inhibits irreversibly ($\gamma$-GCS) $\gamma$-glutamylcysteine synthetase:
↓ [glutathione], co-factor for Se dependent glutathione peroxidase

No effect of BSO itself

BSO ↓ the threshold to observe H$_2$O$_2$-induced DNA strand breaks
$H_2O_2$-induced DNA damage is increased in cases of decreased anti-oxidative defenses

$\blacktriangledown$ BSO $\downarrow$ the threshold to observe $H_2O_2$-induced DNA strand breaks

$\blacktriangledown$ in vivo, mutagenicity of $H_2O_2$ could be increased in cases of deficiency of antioxidant defenses as also suggested in epidemiological studies in cases of Selenium deficiency

How fast is this damage repaired?
Repair of DNA damage in PCCl3 cells: kinetics of DNA repair after irradiation and H$_2$O$_2$

IR 10 Gy

H$_2$O$_2$ degradation by PCCl3 cells

0.2 mM H$_2$O$_2$
H$_2$O$_2$-induced DNA damage is more slowly repaired than that produced after irradiation

- low repair efficiency of DNA strand breaks induced by H$_2$O$_2$
  supports the possible role of H$_2$O$_2$
  in thyroid mutagenesis
DNA damage
> potential carcinogen

\[ H_2O_2 \]

H\(_2\)O\(_2\)-induced DNA damage is ↑ in cases of ↓ anti-oxidative defenses

low repair efficiency of DNA strand breaks induced by \( H_2O_2 \)

Generation of \( H_2O_2 \) necessary to oxidize iodide could induce DNA damage and possibly play a role in thyroid mutagenesis

Chronic endogenous exposure of thyroid cells to \( H_2O_2 \) could be a key to explain high frequency of thyroid tumors and thyroid microcarcinoma
Thanks to

Fonds Erasme pour la recherche médicale, Télévie, FNRS

Dumont J-E.
Vassart G.

Corvilain B.
Miot F.

Bourbonville B.
Burniat A.
De Deken X.
De Graef C.
Ghaddhab C.
Hoste C.
Jin L.
Milenkovic M.
Rigutto S.

Van Sande J.

Chico Galdo V.
Costa M.
Song Y.
Massart C.
Effects of $\text{H}_2\text{O}_2$

- **Low levels** ($\leq 10 \ \mu\text{M}$)
  - Proliferation
  - Cofactor in thyroid hormone synthesis
  - Cuticle formation (insects)
  - NO oxidizer

- **High levels** ($\geq 100 \ \mu\text{M}$)
  - Toxic in defence against pathogens
  - Oxidative stress (proteins, lipids, ..)
  - DNA damage (adducts, SSB, DSB)
  - $\downarrow$
  - MUTAGENESIS
  - $\downarrow$
  - CARCINOGENESIS
  - Apoptosis

- ($\geq 400 \ \mu\text{M}$) Necrosis

- **CHRONIC** SENESCENCE
What happens when protective mechanisms are exceeded?

- ROS
- O₂⁻
- Modulate stress-induced proteins and genes
- Regulate:
  - Cell growth
  - Cell differentiation
  - Death by apoptosis and necrosis
- NOXes
- Superoxide Dismutase

Fenton's reaction:

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \cdot \text{OH} + \text{OH}^{-} \]

Change in DNA conformation:

- Induce lipid peroxidation
- Oxidative damage to proteins
- Decreased efficiency of DNA polymerase and DNA repair
- Activation of signal transduction and cell proliferation
- Inaccurate replication

Chemical changes in bases:

- Enhanced "hot spot" mutagenicity
- Modified H-bonding
- Block in replication

Mutation:

- Oxidation to proteins
- Induced lipid peroxidation
- Change in DNA conformation
- Activation of signal transduction
- Decreased efficiency of DNA polymerase and DNA repair
- Inaccurate replication
<table>
<thead>
<tr>
<th>DISEASE PHENOTYPE</th>
<th>ENZYME DEFECT</th>
<th>MODEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital hypothyroidism</td>
<td>Thyroperoxidase defect, loss of H2O2 inactivation (?) ClO₄ discharge</td>
<td>Human, Goiter hypothyroidism carcinomas,</td>
</tr>
<tr>
<td>Congenital hypothyroidism Goiter, hypothyroidism</td>
<td>DUOX2 defect, loss of H2O2 generation</td>
<td>Human, Goiter, hypothyroidism</td>
</tr>
<tr>
<td>Tumor Rapid cell turnover</td>
<td>TSH receptor defect with lack of Gq activation, α₁R constitutive activation leading to activation of cAMP i.e. cell proliferation, PI₃K/Ca⁺⁺ cascade i.e. H₂O₂, TSH receptor overactivation of Gq generation, apoptosis</td>
<td>? Tgα₁R mice Goiter, hypothyroidism, ClO₄ discharge</td>
</tr>
<tr>
<td>Thyroid tumors ? Frequent thyroid nodules</td>
<td>Phospholipase C cascade, Abnormal DNA repair response ?</td>
<td>Human</td>
</tr>
<tr>
<td>Sporadic papillary carcinoma Papillary carcinoma</td>
<td>H₂O₂, Se deficiency and impaired H₂O₂ catabolism</td>
<td>Human</td>
</tr>
<tr>
<td>Thyroiditis Thyroid necrosis and inflammation</td>
<td>I and Se deficiency, SCN in food, lack of H₂O₂ catabolism</td>
<td>Human</td>
</tr>
<tr>
<td>Myxedematous endemic Atrophy, hypothyroidism cretinism</td>
<td></td>
<td>Rat</td>
</tr>
</tbody>
</table>
Global transcriptional responses to H$_2$O$_2$ at 200$\mu$M and to $\gamma$-radiation at 2.5Gy are similar.

Among all stress agents, the response to H$_2$O$_2$ is most similar to that of $\gamma$-radiation at 2.5Gy.

This suggests that H$_2$O$_2$ and $\gamma$-radiation at 2.5Gy inflict comparable DNA damage.

Detours 2006
118 genes differentially expressed in response to H$_2$O$_2$ at 200µM and to γ-radiation at 2.5Gy accurately classify tumors as CTB or French

<table>
<thead>
<tr>
<th></th>
<th>French error</th>
<th>CTB error</th>
<th>global error</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPLS</td>
<td>8</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>PAM</td>
<td>25</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>RF</td>
<td>42</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>LKSVM</td>
<td>25</td>
<td>7</td>
<td>15</td>
</tr>
</tbody>
</table>

These 118 genes

- Have an expression differing at least by a factor 1.5 between the transcriptional responses to H$_2$O$_2$ and to γ-radiation
- Are not immune system-specific

Detours 2006
A signature of 13 homologous recombination genes accurately classify tumors as CTB or French

<table>
<thead>
<tr>
<th></th>
<th>French error</th>
<th>CTB error</th>
<th>global error</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPLS</td>
<td>17</td>
<td>21</td>
<td>19</td>
<td>0.0038</td>
</tr>
<tr>
<td>PAM</td>
<td>25</td>
<td>21</td>
<td>23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RF</td>
<td>42</td>
<td>21</td>
<td>31</td>
<td>0.063</td>
</tr>
<tr>
<td>LKSVM</td>
<td>8</td>
<td>21</td>
<td>15</td>
<td>0.0038</td>
</tr>
</tbody>
</table>

Signatures representative of 5 DNA repair processes were tested:

1. base excision repair
2. mismatch excision repair
3. nucleotide excision repair
4. homologous recombination
5. non homologous end-joining

The 13 genes involved in homologous recombination lead to accurate classification.

Genes involved in the 4 other repair pathways do not.

Detours 2006
Conclusions

1) $\text{H}_2\text{O}_2$ provokes DNA damages
   > non lethal $[\text{H}_2\text{O}_2]$ (0.05mM to 0.5mM) provoke large number of SSB but also as many DSB as IR in different, *in vitro*, thyroid models
   > potential carcinogen

2) $\text{H}_2\text{O}_2$-induced DNA damages are increased in case of decreased anti-oxidative defences
   > BSO↓ the threshold to observe $\text{H}_2\text{O}_2$-induced DNA strand breaks
   > in vivo, mutagenicity of $\text{H}_2\text{O}_2$ could be increased in case of deficiency of antioxidant defense as also suggested in epidemiological studies in case of Selenium deficiency

3) $\text{H}_2\text{O}_2$-induced DNA damages are slower repaired than those produced after irradiation
   > low repair efficiency of DNA DSB induced by $\text{H}_2\text{O}_2$ support the possible role of $\text{H}_2\text{O}_2$ in thyroid mutagenesis
DNA damage in rat thyroid cell line (PCCl3)

- **Graph 1:**
  - X-axis: Dose (Gy)
  - Y-axis: Score (arbitrary units)
  - Bars for 0, 1-2, 4-5, 10 Gy
  - Significance: *** for 10 Gy
  - Controls (Ctl): 0.1, 1, 5, 10 Gy

- **Graph 2:**
  - X-axis: H₂O₂ (mM)
  - Y-axis: Score (arbitrary units)
  - Bars for 0, 0.01, 0.03, 0.05, 0.1, 0.2, 0.5-0.7, 1 mM
  - Significance: *** for 1 mM

- **Images:**
  - 15 kD γH2AX
    - Ctl 0.1 1 5 10 Gy
  - 15 kD H2AX
  - Ctl 0.1 0.2 0.5 1 mM
  - ctl 1 Gy
  - 5 Gy
  - 10 Gy
  - 0.1 mM 1 mM