A Proteomic Approach to Uncover Neuroprotective Mechanisms of Oleocanthal against Oxidative Stress

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Abstract: Neurodegenerative diseases represent a heterogeneous group of disorders that share common features like abnormal protein aggregation, perturbed Ca\(^{2+}\) homeostasis, excitotoxicity, impairment of mitochondrial functions, apoptosis, inflammation, and oxidative stress. Despite recent advances in the research of biomarkers, early diagnosis, and pharmacotherapy, there are no treatments that can halt the progression of these age-associated neurodegenerative diseases. Numerous epidemiological studies indicate that long-term intake of a Mediterranean diet, characterized by a high consumption of extra virgin olive oil, correlates with better cognition in aged populations. Olive oil phenolic compounds have been demonstrated to have different biological activities like antioxidant, antithrombotic, and anti-inflammatory activities. Oleocanthal, a phenolic component of extra virgin olive oil, is getting more and more scientific attention due to its interesting biological activities. The aim of this research was to characterize the neuroprotective effects of oleocanthal against H\(_2\)O\(_2\)-induced oxidative stress in neuron-like SH-SY5Y cells. Moreover, protein expression profiling, combined with pathways analyses, was used to investigate the molecular events related to the protective effects. Oleocanthal was demonstrated to counteract oxidative stress, increasing cell viability, reducing reactive oxygen species (ROS) production, and increasing reduced glutathione (GSH) intracellular level. Proteomic analysis revealed that oleocanthal significantly modulates 19 proteins in the presence of H\(_2\)O\(_2\). In particular, oleocanthal up-regulated proteins related to the proteasome, the chaperone heat shock protein 90, the glycolytic enzyme pyruvate kinase, and the antioxidant enzyme peroxiredoxin 1. Moreover, oleocanthal protection seems to be mediated by Akt activation. These data offer new insights into the molecular mechanisms behind oleocanthal protection against oxidative stress.

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1. Introduction

With the acceleration of the size of the aging population, the world is now experiencing the “aging era” characterized by an increased incidence of neurodegenerative diseases like Alzheimer’s disease (AD) and Parkinson’s disease (PD) [1]. Neurodegenerative diseases represent a heterogeneous group of disorders whose common characteristic is the progressive and selective loss of neurons. Although many studies have been carried out to understand the pathophysiology of neurodegenerative diseases over the years, more efforts are still needed to uncover the processes that trigger these disorders. Despite different aetiologies, neurodegenerative disorders share common features like abnormal protein aggregation, perturbed Ca$^{2+}$ homeostasis, excitotoxicity, impairment of mitochondrial functions, apoptosis, inflammation, and oxidative stress [2,3]. Oxidative stress is an imbalance between accumulation and removal of reactive oxygen species (ROS). The brain is especially susceptible to oxidative stress due to its high oxygen consumption, high content of oxidizable polyunsaturated fatty acids, and low level of endogenous antioxidants and antioxidant enzymes [4–6]. It has been shown that oxidative stress is involved in the onset and progression of neurodegenerative diseases like PD [2,7–10] and AD [11–13].

Despite recent advances in the research of biomarkers, early diagnosis, and pharmacotherapy, there are no treatments that can halt the progression or reverse the brain changes of any age-associated neurodegenerative diseases. This is likely due to the multifactorial nature of these pathologies that arise from a confluence of multiple, toxic insults.

Diet is considered one of the most important factors in lifestyle. Diet strongly influences the occurrence of cardiovascular and neurodegenerative diseases and, hence, a healthy lifestyle can be related to healthy aging [14]. Numerous epidemiological studies indicate that long-term intake of a Mediterranean diet, characterized by a high consumption of extra virgin olive oil, correlates with better cognition in aged populations [15–17]. Olive oil phenolic compounds have been demonstrated to possess different biological activities like antioxidant [18], antithrombotic [19], and anti-inflammatory [20] activities. Oleocanthal, a dialdehydic form of (−)-deacetoxyligstroside glycoside, is one of the phenolic components of extra virgin olive oil [21]. Even though oleocanthal makes up only 10% of the olive’s phenols, it is getting more and more scientific attention due to its interesting biological activities [22,23]. Oleocanthal is responsible for the bitter and pungent taste of extra virgin olive oil, and has anti-inflammatory properties similar to the nonsteroidal anti-inflammatory drug ibuprofen [24]. In vitro, it has been shown that oleocanthal is effective on the key mediators of AD pathogenesis, amyloid-β and hyper-phosphorylated tau proteins [25–28], which contribute significantly to neurodegeneration and memory loss [29]. Moreover, oleocanthal reduces astrocyte activation and interleukin-1β levels in vivo [21]. Studies on the antioxidant activities of oleocanthal are limited. Only one study carried out in isolated human monocytes showed that oleocanthal inhibits nicotinamide adenine dinucleotide phosphate oxidase (NOX) activity and reduces the intracellular level of superoxide anion [30].

The aim of this research was to characterize the neuroprotective effects of oleocanthal against H$_2$O$_2$-induced oxidative stress in the neuron-like SH-SY5Y cell line. Moreover, protein expression profiling, combined with pathways analyses, was used to investigate the molecular events related to the protective effects, and to gain insight into the underlying mechanisms of neuroprotection and oxidative damage.
2. Results

2.1. Neuroprotective Effects of Oleocanthal against H$_2$O$_2$-Induced Damage

To investigate the potential cytotoxicity of oleocanthal, differentiated SH-SY5Y cells were treated with different concentrations (1–10 μM) of oleocanthal for 24 h (Figure 1a). Of note, oleocanthal did not show any effect on cell viability at any tested concentrations. To study the potential protective activity of oleocanthal against oxidative injury, cells were treated with increasing concentrations (1–10 μM) of oleocanthal before the induction of oxidative stress by 700 μM H$_2$O$_2$ exposure for 1 h (Figure 1c). This peroxide concentration has been chosen as it reduces cell viability by 50% with respect to control cells (Figure 1b). Moreover, similar H$_2$O$_2$ concentrations have been recently used by Piras et al. [31] in differentiated SH-SY5Y cells.

![Figure 1](image)

Figure 1. Viability of differentiated SH-SY5Y treated with oleocanthal in the absence/presence of H$_2$O$_2$. (a) Cells were treated with oleocanthal (1–10 μM) for 24 h, (b) cells were treated with peroxide (0.1–1 mM), or (c) cells were treated with oleocanthal (1–10 μM) and after 24 h exposed to 700 μM H$_2$O$_2$ for 1 h; cell viability was measured by MTT assay. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni’s test. *p < 0.05 with respect to control (CTRL); †p < 0.05 with respect to H$_2$O$_2$.

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. As expected, H$_2$O$_2$ significantly reduced cell viability with respect to control cells. Quantities of 1 to 5 μM oleocanthal were not able to counteract peroxide’s deleterious effects as cell viability was comparable to that of H$_2$O$_2$-treated cells. On the contrary, 10 μM oleocanthal significantly increased cell viability with respect to H$_2$O$_2$-treated cells. For these reasons, the subsequent experiments were carried out using 10 μM oleocanthal.

2.2. Antioxidant Activity of Oleocanthal against H$_2$O$_2$-Induced Oxidative Stress

The ability of oleocanthal to counteract H$_2$O$_2$-induced intracellular ROS production was investigated by the 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay. As illustrated in Figure 2, incubation of differentiated SH-SY5Y cells with H$_2$O$_2$ resulted in a significant and marked increase in intracellular ROS levels. Oleocanthal, in contrast, significantly reduced intracellular ROS levels compared to H$_2$O$_2$. There was no change in ROS levels after treatment with oleocanthal alone.
Figure 2. Antioxidant activity of oleocanthal against H$_2$O$_2$ in differentiated SH-SY5Y cells. Cells were treated with 10 $\mu$M oleocanthal and after 24 h were exposed to H$_2$O$_2$. Intracellular reactive oxygen species (ROS) levels were measured with the peroxide-sensitive probe DCFH-DA as reported in Materials and Methods. Data are expressed as a percentage with respect to H$_2$O$_2$-treated cells. Each bar represents mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni’s test. * $p < 0.05$ with respect to CTRL; $^\circ$ $p < 0.05$ with respect to H$_2$O$_2$.

As GSH is the most abundant endogenous antioxidant [32], we next evaluated the effect of oleocanthal treatment in the absence/presence of H$_2$O$_2$ on intracellular GSH levels by monochlorobimane (MCB) assay (Figure 3). Interestingly, oleocanthal alone was able to increase GSH levels compared with control cells. Exposure to H$_2$O$_2$ caused a significant decrease in GSH levels; meanwhile, pretreatment with oleocanthal significantly increased the amount of GSH compared with in H$_2$O$_2$-treated cells.

Figure 3. Effect of oleocanthal on GSH levels in differentiated SH-SY5Y cells. Cells were treated with 10 $\mu$M oleocanthal and after 24 h were exposed to peroxide. GSH levels were measured using the fluorescence probe MCB as reported in Materials and Methods. Each bar represents the mean ± SEM of four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni’s test. * $p < 0.05$ with respect to CTRL; $^\circ$ $p < 0.05$ with respect to H$_2$O$_2$.

2.3. Protein Expression Analysis

Figure 4 illustrates a representative two-dimensional electrophoresis (2DE) image of differentiated SH-SY5Y human neuroblastoma cellular protein extracts. The quality of the gels was assessed by the software Same Spot which includes the SpotCheck function. SpotCheck is a separate quality control workflow which allowed us to objectively assess the quality of our gels images. We selected
a “gold standard” gel (a reference gel) and each gel was tested against it; the software confirmed that we ran gels in a reproducible manner.

Figure 4. Representative 2D gel map of differentiated SH-SY5Y human neuroblastoma cellular protein extracts. (A) Control cells; (B) H$_2$O$_2$-treated cells; (C) Oleocanthal + H$_2$O$_2$ treated cells. Proteins were separated in a 3–10 nonlinear gradient. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 12% of acrylamide.

Overall, an average of 1100 ± 150 spots was found within a nonlinear pH range from 3 to 10. Normalized spot volumes were analyzed by the ANOVA test to detect the proteins which were significantly ($p < 0.05$, $q$ value $< 0.05$) differentially expressed among different comparisons. Regarding comparison with the control of the 46 spots detected as modified by the treatment with H$_2$O$_2$, 6 were up- and 40 down-regulated. Twelve spots were found differentially expressed in cells treated with oleocanthal; 5 were up- and 7 down-regulated. Finally, of the 46 spots significantly modified by the addition of oleocanthal + H$_2$O$_2$, 14 were up- and 32 down-regulated.

To investigate the protection of oleocanthal against the detrimental effect of H$_2$O$_2$, a comparison between oleocanthal + H$_2$O$_2$ vs. H$_2$O$_2$ was carried out. A volcano plot was constructed to represent fold change in protein expression (Figure 5). By this way, 31 spots were found with significant change of expression; 22 were up- and 9 down-regulated.

Figure 5. Protein expression profiling of oleocanthal + H$_2$O$_2$ vs. H$_2$O$_2$. Scatter plot of fold change (x axis), against log $p$-value (y axis) of all quantified proteins. Up- and Down-regulated proteins are colored red and green, respectively. Dotted line indicates the threshold of significance.

All spots of interest were selected and excised from the gel and identified by nano-LC-ESI-MS/MS. A list of identified proteins, with molecular weight (MW), isoelectric point (pI), score, and coverage values of MS/MS is shown in Table 1, while Tables 2 and 3 show the protein names, ratio, and $p$ values of proteins resulting as significantly changed in the comparison between H$_2$O$_2$ vs. control and oleocanthal + H$_2$O$_2$ vs. H$_2$O$_2$, respectively.
**Table 1.** MS/MS data of protein spots differentially expressed grouped by functional class. MW, molecular weight; pI, isoelectric point; th, theoretical.

| # Spot | Protein Name | Gene  | ID    | Peptide (n) | Unique | Coverage (%) | MW (th) | pI (th) |
|-------|--------------|-------|-------|-------------|--------|--------------|---------|---------|
|       |              |       |       |             |        |              |         |         |
|       | **Metabolism** |       |       |             |        |              |         |         |
| 381   | Guanosine 5'-monophosphate synthase (glutamine-hydrolyzing) | GMPS  | P49915 | 32          | 32     | 52           | 76,716  | 6.42    |
| 521   | Pyruvate kinase PKM | PFKM  | P14618 | 36          | 6      | 59           | 57,937  | 7.96    |
| 559   | Amidophosphoribosyltransferase | PPAT   | Q06203 | 4           | 4      | 10           | 57,398  | 6.3     |
| 693   | Gamma-enolase | ENO2   | P09104 | 13          | 13     | 46           | 47,269  | 4.91    |
| 856   | Cytosolic acyl coenzyme A thioester hydrolase | ACC1T7 | O00154 | 3           | 3      | 14           | 27,041  | 6.51    |
| 876   | Alcohol dehydrogenase (NADP(+)) | AKR1A1 | P14550 | 14          | 14     | 50           | 36,573  | 6.32    |
| 939   | Malate dehydrogenase, cytoplasmic | MDH1   | P40925 | 7           | 7      | 32           | 36,426  | 6.91    |
| 950   | ADP-sugar pyrophosphatase | NUDT5  | Q9UKK9 | 6           | 6      | 26           | 24,328  | 4.87    |
| 1069  | Enoyl-CoA hydratase, mitochondrial | ECHS1  | P30084 | 13          | 13     | 43           | 31,387  | 8.34    |
| 1076  | NADH:ubiquinone oxidoreductase iron-sulfur protein 3, mitochondrial | NDUFS3 | O75489 | 8           | 8      | 32           | 30,242  | 6.98    |
| 1098  | Triosephosphate isomerase | TPI1   | P60174 | 12          | 12     | 57           | 26,669  | 6.45    |
| 2010  | Phosphoglycerate kinase 1 | PGK1   | P00558 | 41          | 35     | 72           | 44,615  | 8.3     |
| 2010  | Isocitrate dehydrogenasemitocondrial isoform2 | IDH2   | P48735 | 25          | 25     | 53           | 45,180  | 7.63    |
|       |              |       |       |             |        |              |         |         |
|       | **Protein synthesis, modification secretion folding** |       |       |             |        |              |         |         |
| 296   | Acylamino-acid-releasing enzyme | APEH   | P13798 | 15          | 15     | 24           | 81,225  | 5.29    |
| 508   | Adenosylhomocysteinase | AHCY   | P23526 | 18          | 18     | 37           | 47,716  | 5.92    |
| 916   | Small glucose-rich tetratricopeptide repeat-containing protein alpha | SGT1  | O43765 | 8           | 8      | 27           | 34,063  | 4.79    |
| 940   | Ubiquitin thioester OTUB1 | OTUB1  | Q96FW1 | 5           | 5      | 23           | 31,284  | 4.85    |
| 1230  | Protein DJ-1 | PARK7  | Q99497 | 11          | 11     | 49           | 19,891  | 6.32    |
| 1812  | Platelet-activating factor acetylhydrolase IB subunit gamma | PAFAH1B3 | Q15102 | 6           | 6      | 27           | 25,734  | 6.33    |
|       |              |       |       |             |        |              |         |         |
|       | **Protein degradation** |       |       |             |        |              |         |         |
| 498   | Ubiquitin carboxyl-terminal hydrolase 14 | USP14 | P54578 | 13          | 13     | 35           | 52,386  | 5.61    |
| 995   | Proteasome activator complex subunit 3 | PSME3 | P61289 | 10          | 10     | 39           | 29,506  | 5.69    |
| 1111  | Ubiquitin carboxyl-terminal hydrolase isozyme L1 | UCHL1 | P09936 | 29          | 29     | 81           | 24,824  | 5.33    |
|       |              |       |       |             |        |              |         |         |
|       | **Cytoskeleton, vesicle motility, transport, vesicle release** |       |       |             |        |              |         |         |
| 430   | Dihydropyrimidinase-related protein 3 | DPYS1L3 | Q14195 | 29          | 24     | 57           | 73,911  | 5.94    |
| 436   | Dihydropyrimidinase-related protein 3 | DPYS1L3 | Q14195 | 30          | 30     | 58           | 73,911  | 5.94    |
| 441   | Dihydropyrimidinase-related protein 3 | DPYS1L3 | Q14195 | 29          | 24     | 58           | 73,911  | 5.94    |
| 1079  | Ran-specific GTPase-activating protein | RANBP1 | P43487 | 9           | 9      | 50           | 23,310  | 5.19    |
| 1425  | Cofilin-1 | CFL1   | P23528 | 8           | 8      | 51           | 18,502  | 8.22    |
| 1743  | F-actin-capping protein subunit β | CAPZB  | P47756 | 17          | 17     | 56           | 30,629  | 5.69    |
## Table 1. Cont.

| # Spot | Protein Name | Gene ID | Peptide (n) | Unique | Coverage (%) | MW (th) | pI (th) |
|--------|--------------|---------|-------------|--------|--------------|---------|---------|
| 590    | ATP-dependent RNA helicase DDX39A | DDX39A | 000148      | 16     | 16           | 35      | 49,130  | 5.46   |
| 621    | Spliceosome RNA helicase DDX39B   | DDX39B | Q13838      | 18     | 18           | 39      | 48,991  | 5.44   |
| 624    | Heterogeneous nuclear ribonucleoprotein H | HNRNPH1 | P31943      | 25     | 13           | 55      | 49,229  | 5.89   |
| 836    | Heterogeneous nuclear ribonucleoprotein A3 | HNRNPA3 | P51991      | 24     | 23           | 49      | 39,595  | 9.1    |
| 876    | Heterogeneous nuclear ribonucleoprotein H3 | HNRNPH3 | P31942      | 12     | 12           | 58      | 36,926  | 6.37   |
| 915    | Heterogeneous nuclear ribonucleoprotein H3 | HNRNPH3 | P31942      | 8      | 8            | 40      | 35,239  | 6.36   |
| 1735   | Heterogeneous nuclear ribonucleoprotein A1 | HNRNPA1 | P09651      | 15     | 11           | 63      | 29,386  | 9.19   |

### Chromatin remodeling and histone

| # Spot | Protein Name | Gene ID | Peptide (n) | Unique | Coverage (%) | MW (th) | pI (th) |
|--------|--------------|---------|-------------|--------|--------------|---------|---------|
| 498    | Poly(U)-binding-splicing factor PUF60 isoform1 | PUF60 | Q9UHX1      | 10     | 10           | 28      | 59,875  | 5.19   |

### Stress response

| # Spot | Protein Name | Gene ID | Peptide (n) | Unique | Coverage (%) | MW (th) | pI (th) |
|--------|--------------|---------|-------------|--------|--------------|---------|---------|
| 181    | 26S proteasome non-ATPase regulatory subunit 1 | PSMD1 | Q99460      | 16     | 16           | 27      | 10,228  | 5.14   |
| 334    | Heat shock protein HSP 90-α | HSP90AA1 | P07900      | 12     | 6            | 19      | 84,660  | 4.94   |
| 335    | Heat shock protein HSP 90-β | HSP90AB1 | P08238      | 15     | 8            | 24      | 83,264  | 4.96   |
| 498    | 60 kDa heat shock protein. mitochondrial | HSPD1 | P10809      | 31     | 31           | 54      | 61,055  | 5.7    |
| 506    | 60 kDa heat shock protein. mitochondrial | HSPD1 | P10809      | 54     | 54           | 73      | 61,055  | 5.7    |
| 1153   | 60 kDa heat shock protein. mitochondrial | HSPD1 | P10809      | 4      | 4            | 35      | 61,055  | 5.7    |
| 701    | DnaJ homolog subfamily A member 2 | DNAJA2 | Q60884      | 15     | 15           | 35      | 45,746  | 6.06   |
| 995    | Proteasome activator complex subunit 3 | PSME3 | P61289      | 10     | 10           | 39      | 29,506  | 5.69   |
| 1087   | Peroxiredoxin-6 | PRDX6 | P30041      | 21     | 21           | 65      | 25,035  | 6      |
| 1090   | Peroxiredoxin-6 | PRDX6 | P30041      | 20     | 20           | 67      | 25,035  | 6      |
| 1122   | Heat shock protein β-1 | HSPB1 | P04792      | 21     | 21           | 77      | 22,783  | 5.98   |
| 1253   | Peroxiredoxin-2 | PRDX2 | P32119      | 30     | 30           | 59      | 21,892  | 5.66   |
| 1269   | Peroxiredoxin-2 | PRDX2 | P32119      | 20     | 20           | 59      | 21,892  | 5.66   |
| 1725   | Peroxiredoxin-4 | PRDX4 | Q13462      | 4      | 4            | 17      | 30,540  | 5.86   |
| 1731   | Peroxiredoxin-1 | PRDX1 | Q06830      | 21     | 21           | 72      | 22,110  | 8.27   |
| 1736   | Peroxiredoxin-1 | PRDX1 | Q06830      | 17     | 17           | 72      | 22,110  | 8.27   |
| 1741   | Thioredoxin-dependent peroxide reductase. mitochondrial | PRDX3 | P30048      | 11     | 11           | 46      | 27,693  | 7.68   |
| 1743   | F-actin-capping protein subunit β | CAPZB | P47756      | 17     | 17           | 56      | 30,629  | 5.69   |

### Miscellaneous

| # Spot | Protein Name | Gene ID | Peptide (n) | Unique | Coverage (%) | MW (th) | pI (th) |
|--------|--------------|---------|-------------|--------|--------------|---------|---------|
| 498    | Ubiquitin carboxyl-terminal hydrolase 14 | USP14 | P54578      | 13     | 13           | 35      | 52,386  | 5.61   |
| 715    | Programmed cell death protein 2-like | PDCD2L | Q9BRP1      | 3      | 3            | 13      | 39,417  | 4.71   |
| 826    | Guanine nucleotide-binding protein G(i) subunit α-2 | GNA12 | P04899      | 16     | 13           | 50      | 40,451  | 5.34   |
| 843    | V-type proton ATPase subunit d 1 | ATP6V1D1 | P61421      | 5      | 5            | 20      | 40,329  | 4.89   |
| 843    | Nucleophosmin | NPM1 | P06748      | 14     | 14           | 52      | 32,575  | 4.64   |
Table 1. Cont.

| # Spot | Protein Name | Gene | ID | Peptide (n) | Unique | Coverage (%) | MW (th) | pI (th) |
|--------|--------------|------|----|-------------|--------|--------------|---------|--------|
| 950    | Annexin A5   | ANXA5| P08758 | 6 | 6 | 18 | 35,937 | 4.93 |
| 1153   | High mobility group protein B1 | HMGB1 | P09429 | 4 | 4 | 24 | 24,894 | 5.6 |
| 1733   | Chloride intracellular channel protein 1 | CLIC1 | O00299 | 16 | 15 | 61 | 26,923 | 5.09 |
| 1743   | N(G).N(G)-dimethylarginine dimethylaminohydrolase 2 | DDAH2 | Q195685 | 13 | 13 | 54 | 29,644 | 5.66 |
| 1767   | MICOS complex subunit MIC60 | IMMT | Q16891 | 41 | 41 | 60 | 82625 | 6.15 |
| 1813   | 28 kDa heat- and acid-stable phosphoprotein | PDAP1 | Q13442 | 3 | 3 | 13 | 20,630 | 8.84 |

Table 2. List of differentially expressed proteins after comparison between H$_2$O$_2$ vs. Control (ctrl). ID: SwissProt accession Number; OD: Normalized optical density.

| # Spot | ID | Gene | Protein Name | Ratio (OD) H$_2$O$_2$/Ctrl | p-Value |
|--------|----|------|--------------|-----------------------------|---------|
| 181    | Q99460 | PSMD1 | 26S proteasome non-ATPase regulatory subunit 1 | 0.76 | 0.026 |
| 335    | P08238 | HSP90AB1 | Heat shock protein HSP 90-beta | 0.67 | 0.018 |
| 381    | P49915 | GMPS | GMP synthase [glutamine-hydrolyzing] | 0.75 | 0.014 |
| 430    | Q14195 | DPYSL3 | Dihydropyrimidinase-related protein 3 | 0.58 | 0.002 |
| 436    | Q14195 | DPYSL3 | Dihydropyrimidinase-related protein 3 | 0.74 | 0.001 |
| 441    | Q14195 | DPYSL3 | Dihydropyrimidinase-related protein 3 | 1.56 | 0.022 |
| 506    | P10809 | HSPD1 | 60 kDa heat shock protein. mitochondrial | 0.83 | 0.011 |
| 559    | Q06203 | PFAT | Amidophosphoribosyltransferase | 0.57 | 0.046 |
| 621    | Q13838 | DDX39B | Spliceosome RNA helicase DDX39B | 0.83 | 0.022 |
| 624    | P31943 | HNRNPH1 | Heterogeneous nuclear ribonucleoprotein H | 0.81 | 0.039 |
| 701    | DNAJ2 | O60884 | DnaJ homolog subfamily A member 2 | 0.77 | 0.017 |
| 708    | P23526 | AHCY | Adenosylhomocysteinase | 0.82 | 0.012 |
| 856    | O00154 | ACOT7 | Cytosolic acyl coenzyme A thioester hydrolase | 0.76 | 0.036 |
| 876    | P14550 | AKR1A1 | Alcohol dehydrogenase (NADP(+)) | 0.69 | 0.043 |
| 876    | P31942 | HNRNPH3 | Heterogeneous nuclear ribonucleoprotein H3 | 0.71 | 0.026 |
| 915    | P31942 | HNRNPH3 | Heterogeneous nuclear ribonucleoprotein H3 | 0.71 | 0.026 |
| 939    | P49025 | MDH1 | Malate dehydrogenase. cytoplasmic | 0.68 | 0.026 |
| 940    | Q96F1W | OTUB1 | Ubiquitin thioesterase OTUB1 | 0.73 | 0.017 |
| 950    | P08758 | ANXA5 | Annexin A5 | 0.78 | 0.012 |
| 950    | Q9UKK9 | NUDT5 | ADP-sugar pyrophosphatase | 0.78 | 0.015 |
| 1069   | P30084 | ECHS1 | Enoyl-CoA hydratase. mitochondrial | 0.78 | 0.023 |
| 1087   | P43487 | RANBP1 | Ran-specific GTPase-activating protein | 0.80 | 0.035 |
| 1090   | P30041 | PRDX6 | Peroxiredoxin-6 | 5.84 | 0.007 |
| 1098   | P60174 | TP1 | Triosephosphate isomerase | 0.68 | 0.004 |
| 1122   | P04792 | HSPB1 | Heat shock protein beta | 0.69 | 0.015 |
Table 2. Cont.

| # Spot | ID      | Gene | Protein Name                                      | Ratio (OD) H$_2$O$_2$/Ctrl | p-Value   |
|--------|---------|------|--------------------------------------------------|-----------------------------|-----------|
| 1179   | P28070  | PSMB4| Proteasome subunit β type-4                       | 0.80                        | 0.036     |
| 1253   | P32119  | PRDX2| Peroxiredoxin-2                                  | 0.17                        | 4.4 x 10^{-7}     |
| 1269   | P32119  | PRDX2| Peroxiredoxin-2                                  | 2.27                        | 3.08 x 10^{-5}    |
| 1725   | Q13162  | PRDX4| Peroxiredoxin-4                                  | 0.62                        | 0.016     |
| 1731   | Q06830  | PRDX1| Peroxiredoxin-1                                  | 0.33                        | 4.28 x 10^{-6}    |
| 1736   | Q06830  | PRDX1| Peroxiredoxin-1                                  | 3.37                        | 1.37 x 10^{-6}    |
| 1741   | P30048  | PRDX3| Thioredoxin-dependent peroxide reductase mitochondrial | 0.48                        | 0.00041   |
| 1743   | P47756  | CAPZB| F-actin-capping protein subunit β                 | 0.80                        | 0.042     |
| 1743   | O95865  | DDAH2| N(G).N(G)-dimethylarginine dimethylaminohydrolase 2 | 1.30                        | 0.021     |
| 1767   | Q16891  | IMMT | MICOS complex subunit MIC60                       | 0.72                        | 0.021     |
| 1812   | Q15102  | PAFAH1B3| Platelet-activating factor acetylhydrolase IB subunit γ | 0.76                        | 0.021     |

Table 3. List of differentially expressed proteins after comparison between oleocanthal (OC) + H$_2$O$_2$ vs. H$_2$O$_2$. ID: SwissProt accession. Number; OD: Normalized optical density.

| # Spot | ID      | Gene      | Protein Name                                      | Ratio M ± SD (OD) OC + H$_2$O$_2$/H$_2$O$_2$ | p-Value   |
|--------|---------|-----------|--------------------------------------------------|---------------------------------------------|-----------|
| 181    | Q99460  | PSMD1     | 26S proteasome non-ATPase regulatory subunit 1    | 1.29                                        | 0.034     |
| 296    | P13798  | APEH      | Acylamino-acid-releasing enzyme                   | 1.28                                        | 0.009     |
| 334    | P08238  | HSP90AB1  | Heat shock protein HSP 90-β                       | 1.33                                        | 0.026     |
| 334    | P07900  | HSP90AA1  | Heat shock protein HSP 90-alpha                   |                                             |           |
| 498    | P54578  | USP14     | Ubiquitin carboxyl-terminal hydrolase 14         | 1.30                                        | 0.023     |
| 498    | Q9UHX1  | PUF60     | Poly(U)-binding-splicing factor PUF60 isoformal1 |                                             |           |
| 521    | P14618  | PKM       | Pyruvate kinase PKM                              | 1.35                                        | 0.035     |
| 590    | O00148  | DDX39A    | ATP-dependent RNA helicase DDX39A                | 1.36                                        | 0.018     |
| 621    | Q13838  | DDX39B    | Spliceosome RNA helicase DDX39B                   | 1.29                                        | 0.013     |
| 715    | Q9BRP1  | PDCD2L    | Programmed cell death protein 2-like             | 0.77                                        | 0.013     |
| 836    | P51991  | HNRNPA3   | Heterogeneous nuclear ribonucleoprotein A3       | 0.64                                        | 0.023     |
| 843    | P61421  | ATP60D1   | V-type proton ATPase subunit d 1                 | 0.64                                        | 0.019     |
| 843    | P06748  | NPM1      | Nucleophosmin                                     |                                             |           |
| 940    | Q96FW1  | OTUB1     | Ubiquitin thioesterase OTUB1                     | 1.45                                        | 0.036     |
| 1109   | P21266  | GSTM3     | Glutathione S-transferase Mu 3                   | 1.23                                        | 0.036     |
| 1111   | P09936  | UCHL1     | Ubiquitin carboxyl-terminal hydrolase isozyme L1 | 1.25                                        | 0.002     |
| 1153   | P09429  | HMG6B1    | High mobility group protein B1                   | 0.43                                        | 0.049     |
| 1179   | P28070  | PSMB4     | Proteasome subunit β type-4                      | 1.30                                        | 0.024     |
| 1736   | Q06830  | PRDX1     | Peroxiredoxin-1                                  | 1.21                                        | 0.029     |
2.4. Pathway and Network Analysis

For the oleocanthal + H$_2$O$_2$ vs. H$_2$O$_2$ comparison, proteins found differentially expressed were included in the Ingenuity Pathways Analysis (IPA) analysis to identify molecular and cellular functions and to investigate whether these proteins work together in specific networks. Therefore, the software generated a main network (Figure 6) with associated biofunctions “Cancer, Organismal Injury, and Abnormalities” and focused on canonical pathways which showed our differentially expressed molecules to fall into functional categories such as protein synthesis, protein degradation, cell death and survival, and cellular function and maintenance. All the proteins found to be up- and down-regulated may concur in an upstream regulator analysis to predict if transcription factors or genes could be activated or inactivated in agreement with the z-score value (z-score > 2 and $p < 0.05$). Two activated transcription regulators were found: MYCN ($z = 2.0$) and NFE2L2 ($z = 2.17$). Furthermore, hypothetical activated and inhibited master regulators in derived causal networks were suggested by the analysis (Table 4).

![Functional Network](image-url)

Figure 6. Functional Network. Proteins differentially expressed resulting from the oleocanthal + H$_2$O$_2$ vs. H$_2$O$_2$ comparison were functionally analyzed through the use of QIAGEN’s Ingenuity Pathway Analysis. Network describes functional relationships among proteins based on known associations in the literature. Solid line: direct interaction; dotted line: indirect interaction. * This protein has been identified in many spots.
Table 4. Master regulators predicted by IPA analysis.

| Master Regulator | Molecule Type                | Predicted Activation State | Activation $z$-Score | $p$-Value  |
|------------------|------------------------------|----------------------------|----------------------|------------|
| GPR68            | G-protein complex           | Inhibited                  | −2.12                | 1.09 × 10^{-4} |
| MARK2            | Kinase                      | Inhibited                  | −2.33                | 2.67 × 10^{-4} |
| miR-149-5p       | Mature microRNA             | Inhibited                  | −2.53                | 1.36 × 10^{-4} |
| ATG7             | Enzyme                      | Inhibited                  | −2.65                | 7.88 × 10^{-6} |
| Smad2/3-Smad4    | Complex                     | Inhibited                  | −2.83                | 3.02 × 10^{-4} |
| PDE2A            | Enzyme                      | Activated                  | +2.53                | 4.07 × 10^{-5} |
| ELMO1            | Other                       | Activated                  | +2.53                | 8.06 × 10^{-5} |
| DOCK5            | Other                       | Activated                  | +2.53                | 8.14 × 10^{-5} |
| FARP2            | Other                       | Activated                  | +2.53                | 1.07 × 10^{-4} |
| ARHGEF6/7        | Group                       | Activated                  | +2.53                | 1.07 × 10^{-5} |
| BAIAIP2          | Kinase                      | Activated                  | +2.33                | 2.44 × 10^{-4} |
| RAC1             | Enzyme                      | Activated                  | +2.33                | 2.47 × 10^{-4} |
| Hif              | Complex                     | Activated                  | +2.33                | 3.28 × 10^{-4} |
| ADORA1           | G-protein complex           | Activated                  | +2.33                | 4.12 × 10^{-4} |
| NOX4             | Enzyme                      | Activated                  | +2.31                | 3.59 × 10^{-6} |
| NFE2L2           | Transcription factor        | Activated                  | +2.23                | 1.07 × 10^{-5} |
| MYCN             | Transcription factor        | Activated                  | +2.00                | 3.85 × 10^{-5} |
| HSF1             | Transcription factor        | Activated                  | +2.00                | 1.33 × 10^{-3} |

2.5. Transcriptional Validation of Proteomic Data Using RT-Polymerase Chain Reaction (PCR) Assays

We selected 9 genes encoding significantly expressed proteins for RT-PCR validation: HSP90AA1, HSP90AB1 which encode for the isoforms $\alpha$ and $\beta$ of the heat shock protein Hsp90; PSMD1, PSMB4, and USP14 which encode for proteasome 26S subunit non-ATPase 1 (Psmd1), proteasome subunit $\beta$ 4 (Psmb4), and ubiquitin carboxyl-terminal hydrolase 14 (Usp14), which are related to the proteasome activity; GSTM3 and PRDX1, which encode for the antioxidant enzymes glutathione S-transferase mu 3 (Gstm3) and peroxiredoxin 1 (Prdx1); and PKM1 and PKM2, which encode for the isoforms 1 and 2 of the glycolytic enzyme pyruvate kinase (Pkm). As shown in Figure 7, except for Gstm3, whose expression was not modulated by oleocanthal in the presence of H$_2$O$_2$, all the other genes were up- or down-regulated in agreement with data reported in Table 2.

2.6. Role of Oleocanthal in the Modulation of the Ubiquitin–Proteasome System

Proteomic and RT-PCR results demonstrated that oleocanthal in the presence of peroxide is able to up-regulate two proteasomal subunits with respect to H$_2$O$_2$, suggesting its positive role in the degradation of misfolded and oxidized proteins; on the other hand, it up-regulates the expression of the de-ubiquitinating enzyme Usp14 that reduces the number of proteins conveyed to the proteasome. To better investigate this conflicting point, we studied the effect of oleocanthal on these three proteins in the absence of oxidative stress by RT-PCR (Figure 8). Interestingly, in the absence of oxidative stress, oleocanthal up-regulated proteasome subunits Psmb4 and Psmd1 but did not influence Usp14 expression.
Figure 7. Transcriptional validation of proteomic data. Differentiated SH-SY5Y were pretreated with 10 μM oleocanthal for 24 h and then exposed to 700 μM H$_2$O$_2$ for 1 h. Total RNA was isolated, and the mRNA level of target genes was quantified using RT-PCR normalized to 18S rRNA reference gene as reported in Materials and Methods. Triplicate reactions were performed for each experiment. Each bar represents the mean ± SEM of three independent experiments. Data were analyzed by Student’s t-test. * $p < 0.05$ with respect to H$_2$O$_2$.

Figure 8. Effect of oleocanthal on the ubiquitin–proteasome system in the absence of H$_2$O$_2$. Differentiated SH-SY5Y were treated with 10 μM oleocanthal for 24 h. Total RNA was isolated, and the mRNA level of target genes was quantified using RT-PCR normalized to 18S rRNA reference gene as reported in Materials and Methods. Triplicate reactions were performed for each experiment. Each bar represents the mean ± SEM of three independent experiments. Data were analyzed by Student’s t-test. * $p < 0.05$ with respect to CTRL.
2.7. Involvement of HSP90 in Oleocanthal Protection against Oxidative Stress

As our proteomic and transcriptional data showed a marked and significant up-regulation of Hsp90α and β by oleocanthal in the presence of H₂O₂ with respect to H₂O₂ alone, we decided to investigate the role of these heat shock proteins in the observed oleocanthal protection against peroxide. A strict correlation between Akt activity and Hsp90 has been demonstrated [33], so we studied the effect of oleocanthal treatment on both Akt activation (phosphorylation) (Figure 9) and Hsp90α and β expression (Figure 10) in the absence/presence of wortmannin, which affects various signal transduction cascades by inhibiting phosphatidylinositol-3-kinases (PI3Ks), thereby blocking Akt phosphorylation. Interestingly, oleocanthal induced a strong activation of Akt (Figure 9). As expected, in the presence of wortmannin the phosphorylated form of Akt was quite undetectable. Oleocanthal treatment was able to significantly up-regulate both Hsp90 isoforms; meanwhile, wortmannin completely abrogated the up-regulation induced by oleocanthal, maintaining Hsp90α and β levels comparable to those in control cells. In order to clarify the involvement of these heat shock proteins in oleocanthal protection against oxidative stress, SH-SY5Y cells were treated with oleocanthal in the absence/presence of wortmannin and exposed to H₂O₂; cell viability was evaluated by MTT assay (Figure 11). Interestingly, in the presence of wortmannin, cell viability of oleocanthal-treated cells was comparable to that of H₂O₂-treated cells, suggesting that Hsp90 and/or Akt are essential to conferring neuroprotection against peroxide.

Figure 9. Akt kinase activation following oleocanthal treatment in the presence of PI3K-specific inhibitor. Differentiated SH-SY5Y were exposed to 100 nM wortmannin 1 h before 10 μM oleocanthal treatment for 1 h. Proteins were separated by SDS-PAGE and immunoblotted for total and phosphorylated form of Akt as reported in Materials and Methods. Each bar represents the mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni’s test. * p < 0.05 with respect to CTRL; § p < 0.05 with respect to oleocanthal.
Figure 10. Akt-specific inhibitor wortmannin prevents oleocanthal-induced HSP90s. Differentiated SH-SY5Y were treated with 100 nM wortmannin 1 h before exposure to 10 μM oleocanthal for 24 h. Total RNA was isolated, and the mRNA level of target genes was quantified using RT-PCR normalized to 18S rRNA reference gene as reported in Materials and Methods. Triplicate reactions were performed for each experiment. Each bar represents the mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni’s test. * \( p < 0.05 \) with respect to CTRL; § \( p < 0.05 \) with respect to oleocanthal; # \( p < 0.05 \) with respect to oleocanthal + wortmannin.

Figure 11. Effect of Akt inhibitor wortmannin on \( \text{H}_2\text{O}_2 \)-induced injury in differentiated SH-SY5Y. Cells were treated with 10 μM oleocanthal for 24 h in absence/presence of 100 nM wortmannin prior to peroxide exposure, then cell viability was assessed with MTT assay. Each bar represents the mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni’s test. * \( p < 0.05 \) with respect to CTRL; * \( p < 0.05 \) with respect to \( \text{H}_2\text{O}_2 \); § \( p < 0.05 \) with respect to oleocanthal.

3. Discussion

In this study, we demonstrated that oleocanthal significantly protects differentiated human neuroblastoma SH-SY5Y cell line from oxidative stress induced by \( \text{H}_2\text{O}_2 \). In particular, oleocanthal reduces intracellular ROS levels and increases endogenous GSH levels. To further elucidate the mechanisms behind oleocanthal’s neuroprotective activity we used proteomic analysis that revealed an involvement of the ubiquitin–proteasome system, heat shock proteins, pyruvate kinases, and peroxiredoxin 1. Proteomic data were validated by real-time PCR analysis in order to have
a complementary and wider vision of oleocanthal effect on both gene and protein expression. Since the levels of transcription of the selected genes were similar to their translation, we considered the comparable change in mRNA levels as a validation of proteomic data. A fundamental issue in the study of nutraceutical compounds is their bioavailability. The pharmacokinetics and bioavailability of oleocanthal have been poorly investigated. Under simulated gastric acid conditions, it has been shown that oleocanthal is stable in acidic conditions at 37 °C and almost half of the oleocanthal diffused from the oil phase into the aqueous solution [34]. Only one study has investigated the bioavailability of oleocanthal by identifying oleocanthal and its metabolites in urines, demonstrating oleocanthal metabolism in the human body [35]. Oleocanthal is mainly metabolized through phase I metabolism, like hydroxylation, hydrogenation, and hydration. Some of the hydrogenated oleocanthal metabolites are further metabolized through phase II metabolism [35]. Even if no studies directly examined the blood–brain barrier (BBB) permeability of oleocanthal, different studies indirectly demonstrated its ability to reach the brain. In particular, Qosa et al. reported a decreased amyloid load in the hippocampus of TgSwDI mice after oleocanthal treatment [21]; meanwhile, in C57BL/6 mice, Abuznait et al. demonstrated that oleocanthal induces P-gp and LRP1, which are responsible for Aβ clearance across the BBB, and increases Aβ degradation by up-regulating Aβ-degrading enzymes [25], strongly suggesting its ability to reach the brain.

The ubiquitin–proteasome system is the main proteolytic complex responsible for the removal of misfolded and damaged intracellular proteins, often produced upon oxidative stress [36]. The proteasome is a non-lysosomal threonine protease [37] that degrades both normal and damaged proteins into short peptides by the internal protease activities [38,39]. It has been observed that the impairment of the ubiquitin–proteasome system is implicated in the pathogenesis of many neurodegenerative diseases [40] like Alzheimer’s disease, Parkinson’s disease, Lewy body dementia, Pick disease, frontotemporal dementia, and Huntington’s disease (HD) [41]. On these bases, the induction of the proteasome system has been emerging as a therapeutic target to counteract these diseases. To our best knowledge, no studies have explored the effect of oleocanthal on the ubiquitin–proteasome system upon oxidative stress. Interestingly, our proteomic data, obtained in the presence of oxidative stress, indicate the up-regulation of two proteasome subunits, namely, the 26S proteasome non-ATPase regulatory subunit and the proteasome subunit β type-4, and the up-regulation of the ubiquitin carboxyl-terminal hydrolases 14 (Usp14). As Usp14 negatively regulates proteasome activity by ubiquitin chain disassembly as well as by a noncatalytic mechanism [42], oleocanthal, in the presence of oxidative stress, has two opposite effects: on one side, it up-regulates the proteasome and, on the other side, it up-regulates Usp14, abrogating the suggested enhancement of the proteasome activity. For this reason, we examined the effect of oleocanthal in the absence of oxidative stress by real-time PCR. Intriguingly, in normal conditions, oleocanthal is able to induce proteasome activity as it up-regulates proteasome subunits Psmd1 and Psmb4 and does not affect Usp14 expression, suggesting that oleocanthal could have a role in the degradation of misfolded proteins rather than in the degradation of oxidized ones. Our observations are in agreement with different studies that demonstrated that oleocanthal counteracts neurodegenerative diseases by enhancing the clearance of misfolded proteins like amyloid-β, phosphorylated tau, and α-synuclein [27,43,44].

Hsp90 is a chaperone protein important for maintaining stability, maturation, and signaling of Hsp90 client proteins [45]. It has been demonstrated that Hsp90 maintains cell proliferation and cell survival by different mechanisms. Hsp90 may inhibit apoptosis by binding to apoptotic peptidase-activating factor 1, resulting in the prevention of apoptosome formation, caspase activation, and apoptotic cell death [46]. In addition, Taiyab et al. showed that inhibition of Hsp90 results in ER stress-induced apoptosis in rat histiocytoma [47]. A strict correlation between Hsp90 and Akt has been demonstrated [33], as the inhibition of the binding of Akt to Hsp90 inactivates Akt and makes cells more susceptible to apoptosis-inducing stimuli [48]. Interestingly, Xie et al. observed that the inhibition of Akt activity led to a reduction of Hsp90 level, suggesting that Akt activation can elevate Hsp90 protein levels [49]. Our data demonstrated that oleocanthal, upon oxidative stress, is able to
up-regulate Hsp90. As Hsp90 acts with Akt, we also evaluated the effect of oleocanthal treatment on Akt activation (phosphorylation). As expected, we measured a strong and significative activation of Akt. Moreover, Akt inhibition by wortmannin led to the inhibition of the up-regulation of Hsp90 induced by oleocanthal, in agreement with the previous observation by Xie et al. Of note, the fact that wortmannin totally abrogated oleocanthal protection against oxidative stress suggests a fundamental role of Akt and/or Hsp90 in the protective mechanisms elicited by oleocanthal against oxidative stress in SH-SY5Y neuronal cells. As wortmannin can affect other signaling cascades apart from Akt, we could not exclude that oleocanthal regulates neuroprotective effects through other signaling pathways. Further studies are necessary to fully elucidate this aspect.

Our proteomic and expression data indicated a significative up-regulation of Pkm1 and Pkm2 by oleocanthal in the presence of oxidative stress. Pyruvate kinase, catalyzing the final rate-limiting step in glycolysis, is a crucial enzyme for glucose metabolism and energy production in the brain. Elevated levels of pyruvate kinase have been suggested to be protective in neurodegeneration as decreased aerobic glycolysis in the brain leads to a loss of cell survival mechanisms that counter pathogenic processes underlying neurodegeneration [50]. Moreover, Luo et al. demonstrated that Pkm hinders the Aβ fibrillation and reduces the toxicity of Aβ aggregates in SH-SY5Y cells, suggesting that Pkm interferes with the stability of the Aβ oligomer by hydrophobic and hydrophilic interactions of its surface [51].

Oleocanthal also induced a strong up-regulation of the antioxidant enzyme Prdx1 in the presence of H₂O₂, as indicated by our proteomic and expression results. Peroxiredoxins play an important role in cell proliferation, redox signaling, differentiation, and gene expression [52,53]. In particular, Prdx1 eliminates hydrogen peroxide produced during cellular metabolism [54] and participates in cell survival by enhancing the expression of the pro-survival factor protein kinase B (PKB) [55]. The role of Prdx1 in AD has been recently highlighted by Majd et al. who observed reduced levels of Prdx1 and 2 in postmortem brains of AD [56]; meanwhile, Schreibelt et al. analyzed the functional role of Prdx1 using a brain endothelial cell line overexpressing Prdx1 and showed that enhanced Prdx1 expression in brain endothelial cells increased BBB integrity and reduced monocye adhesion to and migration across a brain endothelial cell layer [57].

Focusing on proteomic results, the quantitative two-dimensional analysis showed that the insult induced by peroxide induced a significantly different expression of four Prdx in the human neuroblatoma SH-SY5Y cell line. Interestingly, two near spots, different for the pI value and for level of expression, were detected for PRDX1, 2, and 6 in our samples, suggesting the presence of post-translational modification. Post-translational modification and intracellular protein–protein interactions have the potential to influence Prdx catalytic activity and susceptibility to hyperoxidation [58]. We found for all three Prdx an increase of expression of more acidic spots and a significant reduction of more basic spots in the H₂O₂ sample with respect to the control. The protective effect of oleocanthal takes place by way of an increase of more acidic spots of Prx1. The nature of this modification needs to be investigated and underlines a mechanism of control of protein activity.

Finally, all proteins found differentially expressed from a comparison of oleocanthal + H₂O₂ vs. H₂O₂ were analyzed by IPA to explore in depth the network, functions, and molecules (e.g., upstream regulators) that could play a role in the protection induced by oleocanthal. The molecular functions defined by our differentially expressed proteins were well arranged in protein synthesis, cell death and survival, protein degradation, and cellular function and maintenance. On the other hand, the up-regulation of heat shock protein and proteasome members in addition to ubiquitin enzymes and pyruvate kinase concurred to predict cellular viability and vitality (Figure 12). As concerns upstream regulators, intriguing results arose from the IPA analysis. Along the list of noteworthy potential activated or inhibited upstreams were NFE2L2, HSF1, and ATF7.
NFE2L2, which encodes for the transcription factor Nrf2, is important for the coordinated up-regulation of genes in response to oxidative stress. Very recent studies showed neuroprotective actions [59,60] through the activation of Akt/Nrf2/antioxidant enzymes in neuronal cells. On the other hand, the significant up-regulation of the active form of Akt that we found in our results agrees with an activation state of Nrf2.

HSF1 works as a stress-inducible and DNA-binding transcription factor that plays a central role in the transcriptional activation of the heat shock response, leading to the expression of a large number of chaperone heat shock proteins. A central role of HSF1 in synaptic fidelity and memory consolidation has been recently suggested by Hooper and coworkers [61] who showed that the activation of HSF1 alone augmented vesicle transport and synaptic scaffolding proteins. Meanwhile, HSF1 agonists can improve cognitive function in dementia models and activation of neuroprotective signaling pathways. Finally, HSF1 protected neurons from death caused by accumulation of misfolded proteins. This neuroprotection result was abrogated by inhibition of classical deacetylases such as SIRT1 [62].

As concerns ATG7 (autophagy-related 7), a predicted inhibition was advanced by IPA analysis. ATG7 is an E1-like activating enzyme involved in the 2-ubiquitin-like systems required for cytoplasm-to-vacuole transport and autophagy. Recent findings reveal that selective neuronal deletion of ATG7 is strongly protective against neuronal death and overall brain hypoxic–ischemic injury [63].

Overall, our results, combined with IPA analysis, suggest that the major part of protein targets of oleocanthal in response to peroxide insult belongs to the proteostasis network including proteasome, ubiquitin, and chaperone proteins which move to restore equilibrium between protein synthesis, folding, trafficking, secretion, and degradation in different cell compartments. Moreover, our findings add new evidence on the effects of oleocanthal on heat shock protein 90, Pkm1 and 2, and Prdx1.

4. Materials and Methods

4.1. Materials

MTT, DCFH-DA, H$_2$O$_2$, dimethyl sulfoxide (DMSO), MCB, Phosphate-Buffered Saline (PBS), bovine serum albumin (BSA) Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, wortmannin, primers listed in Table 5, mammalian protease inhibitor mixture, radioimmunoprecipitation assay (RIPA) buffer, all trans retinoic acid (RA), and all other chemicals of the highest analytical grade were purchased from Sigma Chemical (St. Louis, MO, USA). PhosSTOP was purchased from Roche Diagnostics (Mannheim, Germany).
Table 5. List of primers for real-time PCR.

| Gene      | 5′-Forward-3′                                      | 5′-Reverse-3′                                 |
|-----------|---------------------------------------------------|-----------------------------------------------|
| GSTM3     | TTGAGGCTTTGGAGAAAAATC                             | TGAAAAAGCIAAGCAAGAGGAG                        |
| HSP90AA1  | ATATCACAGGTTGACGACAAAG                            | GTGAACGACTGACTAAGAGTCTCTCC                   |
| HSP90AB1  | TCTATTACATCTAAGTTGAGAG                            | CTCTTCCATCAAATCTCTTGT                        |
| PSMB4     | CCGCTGATTTCAGTATTTG                               | CCAATGAAATAGCTACTGCTTTG                      |
| PSMD1     | CCCAGTTATTGGATAACCCAG                             | CTCCGAGAGAGAGGTGTTC                          |
| USP14     | ACCCCCTAGAGATTGTTTGT                              | ATCTGGATAGATGACTGCTCC                       |
| PKM1      | GAGCCAGGCTTCCGTCACC                              | TGCCAGACTCTCGGACAGACT                      |
| PKM2      | CAGAGGCTTCCATGCACCCACTAC                          | CAGACTGAGAGAGATGCTTCTCT                     |
| PRDX1     | GGGTCAATACACCTTTAAGAAC                            | CTTCATGAGCTTTTAAAGCC                       |
| 18S rRNA  | CAGGAGGATGAAAAGGATG                               | TATTTCTTCTTGAGACACC                        |

4.2. Cell Culture and Treatments

The SH-SY5Y human neuroblastoma cell line was obtained from Sigma-Aldrich (Milan, Italy). Cells were grown in DMEM supplemented with 10% (v/v) of FBS, 2 mM glutamine, 50 U/mL of penicillin, and 50 μg/mL of streptomycin and maintained at 37 °C in a humidified incubator with 5% CO₂ as previously reported [64]. Cell differentiation was induced, reducing serum levels of the medium to 1% with 10 μM of RA for seven days prior to treatments. Differentiated SH-SY5Y were treated with oleocanthal for 1 or 24 h. Oleocanthal was dissolved in DMSO and kept at −20 °C until use. The control group was treated with an equivalent volume of the vehicle alone. Oxidative stress was induced by exposing cells to 700 μM H₂O₂.

4.3. MTT Assay

Cells were treated with different concentrations of oleocanthal (1–10 μM) for 24 h prior to induction of oxidative stress. Cell viability was evaluated by measuring formazan formation as previously reported [64,65]. Briefly, after treatments, cells were incubated with 0.5 mg/mL of MTT solution for 1 h at 37 °C. After incubation, MTT was removed and 100 μL of DMSO were added, and the absorbance was recorded at λ = 595 nm using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; PerkinElmer, Wellesley, MA, USA).

4.4. Intracellular ROS Production Assay

The production of intracellular reactive oxygen species was evaluated using the fluorescent probe DCFH-DA as reported in [66]. Briefly, differentiated SH-SY5Y were treated with oleocanthal for 24 h and then incubated with 10 μM DCFH-DA in DMEM w/o FBS for 30 min. After DCFH-DA removal, cells were incubated with H₂O₂ for 30 min. Cells were washed with PBS and the fluorescence was measured using 485 nm excitation and 535 nm emission with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, PerkinElmer, Wellesley, MA, USA).

4.5. Intracellular GSH Levels Assay

The levels of reduced glutathione (GSH) were evaluated by a fluorometric assay, using the fluorescent probe MCB [64,67]. Briefly, differentiated SH-SY5Y pretreated with oleocanthal were exposed to H₂O₂ for 1 h and incubated with 50 μM MCB in serum-free medium for 30 min at 37 °C. After incubation, cells were washed in PBS and the fluorescence was measured at 355 nm (excitation) and 460 nm (emission) with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter; PerkinElmer, Wellesley, MA, USA).
4.6. RNA Extraction

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer’s protocol. The yield and purity of the RNA were measured using a NanoVue Spectrophotometer (GE Healthcare, Milano, Italy).

4.7. Analysis of mRNA Levels by Reverse Transcriptase Polymerase Chain Reaction

cDNA was obtained by reverse transcribing mRNA starting from 1 µg of total RNA using an iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer’s protocol. The subsequent polymerase chain reaction (PCR) was performed in a total volume of 10 µL containing 2.5 µL (12.5 ng) of cDNA, 5 µL SsoAdvanced Universal SYBR Green Supermix (BIO-RAD), and 0.5 µL (500 nM) of each primer. The primers used are reported in Table 5; 18S rRNA was used as reference gene.

4.8. Western Blotting

After treatments, differentiated SH-SY5Y were collected and homogenized in RIPA buffer with a mammalian protease inhibitor mixture and PhosSTOP. Samples were boiled for 5 min prior to separation on 10% MiniPROTEAN TGX Precast Protein Gels (BIO-RAD, Hercules, CA, USA). The proteins were transferred to a nitrocellulose membrane (Hybond-C; GE Healthcare, Buckinghamshire, UK) in Tris-glycine buffer at 110 V for 90 min. Membranes were then incubated in a blocking buffer containing 5% (w/v) skimmed milk and incubated with anti-phospho-Akt, anti-Akt (Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin (Sigma-Aldrich), which was used as internal normalizer, overnight at 4 °C on a three-dimensional rocking table. The results were visualized by chemiluminescence using Clarity Western Enhanced Chemiluminescence (ECL) reagent according to the manufacturer’s protocol (BIO-RAD). Semiquantitative analysis of specific immuno-labeled bands was performed using ImageLabTM 5.2 Software (BIO-RAD).

4.9. Sample Preparation for Proteomic Analysis

For proteomic analysis, differentiated SH-SY5Y cells (about \(7 \times 10^5\) cells) were treated with oleocanthal or \(H_2O_2\), or with both for 24 h as described above. At the end of treatment, cells were collected and washed with PBS. After centrifugation (1000 \(\times\) g for 5 min), the resulting pellets were immediately frozen and stored at \(-80\) °C until use. For proteomic studies, each condition was performed in triplicate.

4.10. 2DE Analysis

Cell pellets were resuspended in rehydration solution [68], and protein contents of resulting protein extracts were measured with an RC-DC Protein Assay from Bio-Rad.

2DE was carried out as previously described [69]. Briefly, 200 µg of proteins were filled up to 450 µl in rehydration solution. Immobiline Dry-Strips (GE Health Care Europe; Uppsala, Sweden); 18 cm, nonlinear gradient pH 3–10) were rehydrated overnight in the sample and then transferred to the Ettan IPGphor Cup Loading Manifold (GE Healthcare) for isoelectrofocusing (IEF). The second dimension (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; SDS-PAGE) was carried out by transferring the proteins to 12% polyacrylamide, running at 16 mA per gel and 10 °C for about 16 h, using the Protean® Plus Dodeca Cell (Bio-Rad). The gels were stained with Ruthenium II tris (bathophenanthroline disulfonate) tetrasodium salt (SunaTech Inc., Suzhou, China) (RuBP). ImageQuant LAS4010 (GE Health Care) was used for the acquisition of images. The analysis of images was performed using Same Spot (v4.1, TotalLab, Newcastle Upon Tyne, UK) software. The spot volume ratios between the four different conditions were calculated using the average spot normalized volume of the three biological replicates performed in duplicate. The software included statistical analysis calculations.
4.11. 2DE Statistical Analysis

A comparison among the different treatments was performed. The significance of the differences of normalized volume for each spot was calculated by the software Same Spot including the analysis of variance (ANOVA test). The protein spots of interest were cut out from the gel and identified by LC-MS analysis.

4.12. Spot Digestion and Protein Identification

The gel pieces were washed twice with the wash buffer (25 mM NH₄HCO₃ in 50% acetonitrile (ACN)). Afterwards, proteins were reduced with 10 mM dithiothreitol (DTT) (45 min, 56 °C), and alkylated with 55 mM iodoaceticamide (IAA) (30 min, RT, in the dark). After two washes with the wash buffer, spots were completely dried in a centrifrap vacuum centrifuge. The dried pieces of gel were rehydrated for 30 min at 4 °C in 30 µL of trypsin porcine (Promega, Madison, WI, USA) solution (3 ng/µL in 100 mM NH₄HCO₃) and then incubated at 37 °C overnight. The reaction was stopped by adding 10% trifluoroacetic acid (TFA). Samples were analyzed by LC-MS as described [70] using a shorter chromatographic gradient and in autoMS mode. Each digested spot sample was analyzed by LC-MS/MS using a Proxeon EASY-nLCII (Thermo Fisher Scientific, Milan, Italy) chromatographic system coupled to a Maxis HD UHR-TOF (Bruker Daltonics GmbH, Bremen, Germany) mass spectrometer. Peptides were loaded on the EASY-Column TM C18 trapping column (2 cm L., 100 µm I.D, 5 µm ps, Thermo Fisher Scientific), and subsequently separated on an Acclaim PepMap100 C18 (75 μm I.D., 25 cm L, 5 µm ps, Thermo Fisher Scientific) nanoscale chromatographic column. The flow rate was set to 300 nL/min and the gradient was from 2% to 20% of B in 12 min followed by 20 to 45% in 9 min and from 45% to 90% in 2 min (total run time 35 min). Mobile phase A was 0.1% formic acid in H₂O and mobile phase B was 0.1% formic acid in acetonitrile. The mass spectrometer, typically providing a 60,000 full width at half maximum (FMHW) resolution throughout the mass range, was equipped with a nanoESI spray source. The mass spectrometer was operated in Data Dependent Acquisition mode (DDA), using N₂ as the collision gas for collision-induced dissociation (CID) fragmentation. Precursors in the range 400 to 2200 m/z (excluding 1220.0–1224.5 m/z) with a preferred charge state +2 to +5 and absolute intensity above 4706 counts were selected for fragmentation in a fixed cycle time of 3 s. After two spectra, the precursors were actively excluded from selection for 36 s. Isolation width and collision energy for MS/MS fragmentation were set according to the mass and charge state of the precursor ions (from 2+ to 5+ and from 21 to 55 eV). In-source reference lock mass (1221.9906 m/z) was acquired online throughout runs.

The raw data were processed using PEAKS Studio v7.5 software (Bioinformatic Solutions Inc., Waterloo, ON, Canada) using the function “correct precursor only”. The mass lists were searched against the nextprot database including isoforms (version as of June 2017; 42,151 entries) using 10 ppm and 0.05 Da as the highest error tolerances for parent and fragment ions, respectively. Carbamidomethylation of cysteines was selected as fixed modification and oxidation of methionines and deamidation of asparagine and glutamine as variable modifications allowing 2 missed cleavages.

4.13. Network Analysis

Proteins differentially expressed obtained from the oleocanthal + H₂O₂ vs. H₂O₂ comparison were functionally analyzed through the use of QIAGEN’s Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, CA, USA, www.qiagen.com/ingenuity) with the aim to determine the predominant canonical pathways and interaction network involved. Swiss-Prot accession numbers and official gene symbols were inserted into the software along with corresponding comparison ratios and p values. The network proteins associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. The created networks describe functional relationships among proteins based on known associations in the literature. A comparison of the different analyses was created and the upstream regulators whose activity appears to change in
a significant manner according to the activation z-score value were shown. Finally, to generate plausible causal networks which explain observed expression changes, hidden connections in upstream regulators were also uncovered.

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**Abbreviations**

| Acronym | Description |
|---------|-------------|
| 2DE     | Two-dimensional electrophoresis |
| AD      | Alzheimer’s disease |
| ANOVA   | Analysis of variance |
| BBB     | Blood–brain barrier |
| BSA     | Bovine serum albumin |
| CID     | Collision-induced dissociation |
| DCFH-DA | 2,7-Dichlorodihydrofluorescein diacetate |
| DDA     | Data dependent acquisition mode |
| DMEM    | Dulbecco’s modified Eagle’s medium |
| DMSO    | Dimethyl sulfoxide |
| DTT     | Dithiothreitol |
| ECL     | Enhanced chemiluminescence |
| FMHW    | Full width at half maximum |
| GSH     | Reduced glutathione |
| Gstm3   | Glutathione S-transferase mu 3 |
| IEF     | Isoelectrofocusing |
| IPA     | Ingenuity pathways analysis |
| MCB     | Monochlorobimane |
| MTT     | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide |
| NOX     | Nicotinamide adenine dinucleotide phosphate oxidase |
| PBS     | Phosphate-buffered saline |
| PCR     | Polymerase chain reaction |
| PD      | Parkinson’s disease |
| Prdx    | Peroxiredoxin |
| Prk     | Pyruvate kinase |
| Psmb4   | Proteasome subunit β 4 |
| Psmd1   | Proteasome 26S subunit non-ATPase 1 |
| RA      | All trans retinoic acid |
| RIPA    | Radioimmunoprecipitation assay |
| ROS     | Reactive oxygen species |
| SDS-PAGE| Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| Usp14   | Ubiquitin carboxyl-terminal hydrolase 14 |

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