Salivary proteome changes in response to social anxiety and sensory stimuli

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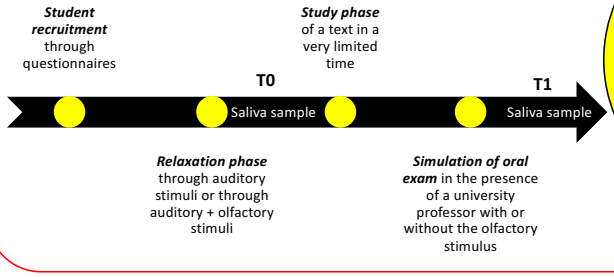
Introduction

Stress is an environmental and psychological stimulus, which creates mental and physiological responses, eventually leading to pathological conditions. Mild stress can be useful for cognitive tasks and performance while constant and high stress leads to anxiety and depression. From the biochemical point of view, during stress episodes the involvement of the hypothalamic-pituitary-adrenal axis, adrenergic sympathetic, and immune systems has been observed. These three systems have a common characteristic, which is their secretory function. For this reason, to evaluate their activity, it is possible to measure their products in blood and other biological fluids. Indeed, during a stress conditions, variations of catecholamine, cortisol and protein levels in body fluids can be detected. Some studies have shown a significant increase of salivary α-amylase and a decrease of salivary IgA as consequence of a stress. An analysis of salivary proteins can be quite useful to measure pathophysiological modifications since sample collection is less invasive than blood sampling and therefore less stressful for the individual.

Stress test



Aim

The aim of this study was to analyze the salivary proteome of university students , which underwent a stress condition , to obtain a better knowledge of the molecular mechanisms involved in the pathophysiological responses.

Methods

- University students (18 to 30 years old) were selected by a specific questionnaire.
- Enrolled students underwent a relaxation phase through auditory or auditory plus olfactory sensory stimuli and then a stress phase with or without the olfactory stimulus.
- The stress phase was divided into:
  - a study phase of a text within a limited time;
  - an exam in the presence of a university professor.
- During the stress test, electrocardiogram, and skin conductance were constantly monitored.
- Whole saliva samples were collected at the end of the relaxation (T0) and stress phases (T1), immediately processed and stored at -80°C until use.

- 2-DE was carried out on 200 μg of proteins using 18 cm, linear gradient pH 3–10 Immobiline Dry-Strips; SDS-PAGE was carried out on 12.5% polyacrylamide gels.
- Gels were stained with Ruthenium complex
- Differentially expressed proteins were identified by LC-MS/MS
- Western Blot was performed to validate 2DE results (3 μg of protein for α-Amylase, 5 μg of protein for IGHA1)

Results RR and Skin Conductance

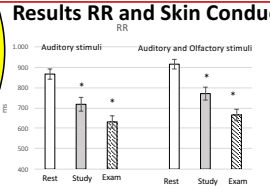


Figure 1 – Graphical representation of the values of mean RR during the different phases of the stress test.

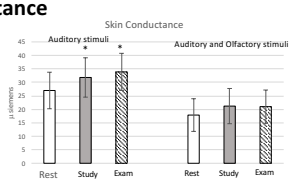


Figure 2 – Graphical representation of the values of skin conductance in the three phases of the stress test.

Results 2D-Electrophoresis

#	Protein	MW	pI	p-Value	Ratio (T1/T0)
67, 69	Polymeric immunoglobulin receptor	83284	5.59	0.0302, 0.0437	0.80, 0.86
98, 100	Ig mu chain C region	49307	6.35	0.0436, 0,0084	0.75, 0.75
102, 105	Serotransferrin	77064	6.7	0.0141, 0.0462	0.77, 0.80
126, 128	Heat shock cognate 71 kDa protein	70898	5.37	0.003, 0,0058	0.77, 0.69
170	Serum albumin	69367	5.67	0.0084	0.66
186	Heat shock 70 kDa protein 1A, 1B	69921, 69921	5.48, 5.48	0.0176	0.67
187	Serum albumin	69367	5.67	0.0082	0.71
211	Plastin-2	70289	5.29	0.0185	0.57
271, 339, 348	Alpha-amylase, Iso 1, 2B	57768, 57710	6.34. 6.49	0.0009, 0.0047, 0.0001	1.30, 1.34, 1.39
364, 368, 369, 370, 373, 374, 375, 376, 1148	Ig gamma chain C region	36106, 35901	8.46, 7.66	0.0001, 0.0039, 0.0041, 0.0074, 0.0008, 0.0006, 0.0124, 0.0007, 0.0044	0.53, 0.68, 0.58, 0.66, 0.49, 0.52, 0.59, 0.53, 0.80
457, 461, 464	Actin, cytoplasmic Iso 1, 2	41737, 41793	5.29. 5.31	0.0074, 0.0308, 0.0215	0.75, 0.79, 0.75
487	Leukocyte elastase inhibitor	42742	5.90	0.024	0.698
539	Complement C3c a-chain fragment	39488	4.79	0.0187	0.62
558, 559, 1149	Glyceraldehyde-3-phosphate dehydrogenase	35922	8.58	0.0178, 0.0024, 0.0198	0.47, 0.59, 0.47
582	Malate dehydrogenase	36426	6.89	0.0491	0.75
585, 612	Zinc-alpha-2-glycoprotein	34259	5.58	0.019, 0.024	0.63, 0.57
590	L-lactate dehydrogenase A chain	36558	8.46	0.04	0.46
636	Cysteine-rich secretory protein 3	27630	8.11	0.0027	0.62
692	14-3-3 protein zeta/delta	27745	4.73	0.0129	0.61
733	Rho GDP-dissociation inhibitor 1	23207	5.01	0.0007	0.65
745	Rho GDP-dissociation inhibitor 2	22988	5.08	0.007	0.53
756	Immunoglobulin J chain	18099	5.09	0.0251	0.78
770, 771	Glutathione S-transferase P	23356	5.44	0.0127, 0.0262	0.65, 0.67
945	Prolactin inducible protein	13523	5.40	0.0163	0.77
1015	Calgranulin B	13241	5.71	0.0295	0.698
1018	Cystatin SA	14350	4.85	0.0475	1.433
1020	Cystatin S	14189	4.83	0.0443	1.304
III, IV, V, VI, VII, VIII, IX, X, XI, XIII, XIV	Ig kappa chain C region	11609	6.11	0.0099, 0.0125, 0.0128, 0.0078, 0.0033, 0.0065, 0.0033, 0.0142, 0.0042, 0.0009, 0.0031	0.72, 0.74, 0.77, 0.76, 0.76, 0.78, 0.68, 0.70, 0.78, 0.78, 0.67
III, IV, V, VI, VII, VIII, IX, X, XI, XIII, XIV	Ig lambda chain C regions, Iso 2, 3	11294, 11237	6.91. 6.91	0.0099, 0.0125, 0.0128, 0.0078, 0.0033, 0.0065, 0.0033, 0.0142, 0.0042, 0.0009, 0.0031	0.72, 0.74, 0.77, 0.76, 0.76, 0.78, 0.68, 0.70, 0.78, 0.78, 0.67

Panel 1 - List of significant differentially expressed proteins in the Auditory stimuli group (T1 vs T0) (p value < 0.05)

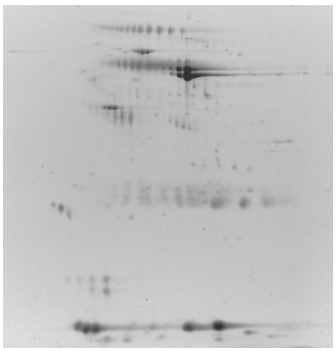


Figure 3 – A representative 2-DE gel image of salivary protein

#	Protein	MW	pI	p-Value	Ratio (T1/T0)
75, 78, 81	Polymeric immunoglobulin receptor	83284	5,59	0.04, 0.001, 0.028	0.83, 0.78, 0.86
170, 192	Serum albumin	69367	5,67	0.027, 0.024	0.832, 0.77
216, 246, 247, 250	Ig alpha chain C, Iso 1, 2	37655, 36526	6,08, 5,86	0.005, 0.031, 0.005, 0.001	0.84, 0.86, 0.74, 0.83
271, 348	Alpha-amylase, Iso 1, 2B	57768, 57710	6,34, 6,49	0.038, 0.003	1.16, 1.21
572	Unknown protein			0.05	1358,00
699	Unknown protein			0.048	0.813
733	Rho GDP-dissociation inhibitor 1	23207	5,01	0.019	0.801
756, 827	Immunoglobulin J chain	18099	4,59	0.039, 0.018	0.78, 0.72
839	Phosphatidylethanolamine-binding protein 1	21057	7,43	0.042	0.737
1010	Fatty acid-binding protein, epidermal	Q01469	15033	6.82	1703,00
C	Fructose-bisphosphate aldolase A	39420	8,39	0.021	0.688
I, II, III, IV, V, VI, XI, XIII	Ig kappa chain C region	11609	6,11	0.002, 0.015, 0.022, 0.002, 0.005, 0.021, 0.01, 0.019	0.72, 0.78, 0.79, 0.81, 0.79, 0.82, 0.82, 0.83
I, II, III, IV, V, VI, XI, XIII	Ig lambda chain C regions, Iso 2, 3	11294, 11237	6.91, 6.91	0.002, 0.015, 0.022, 0.002, 0.005, 0.021, 0.01, 0.019	0.72, 0.78, 0.79, 0.81, 0.79, 0.82, 0.82, 0.83

Panel 2 - List of significant differentially expressed proteins in Auditory and Olfactory stimuli group (T1 vs T0) (p value < 0.05)

Western Blot Validation

α-Amylase – Auditory stimuli (p-value = 0,0048)

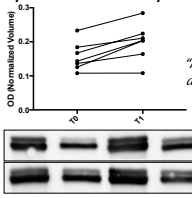
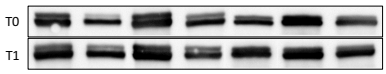


Figure 4 – Graphical representation of western blot validation, performed on "Auditory Stimuli" group with antibody anti-α-Amylase. The normalized optical densities were analysed with paired t-Student test



α-Amylase – Auditory and Olfactory stimuli (p-value = 0,0477)

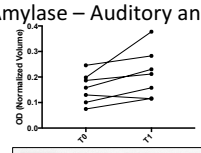
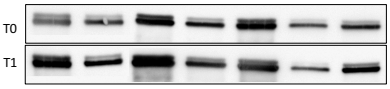


Figure 5 – Graphical representation of western blot validation, performed on "Auditory and Olfactory Stimuli" group with antibody anti-α-Amylase. The normalized optical densities were analysed with paired t-Student test



IGHA1 – Auditory stimuli (p-value = 0,0001)

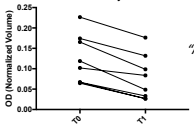


Figure 6 – Graphical representation of western blot validation, performed on "Auditory Stimuli" group with antibody anti-IGHA1. The normalized optical densities were analysed with paired t-Student test

IGHA1 – Auditory and Olfactory stimuli (p-value = 0,0126)

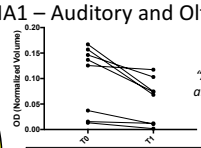
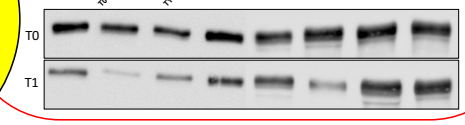


Figure 7 – Graphical representation of western blot validation, performed on "Auditory and Olfactory Stimuli" group with antibody anti-IGHA1. The normalized optical densities were analysed with paired t-Student test



Conclusion

In conclusion, we observed variations of protein secretion despite the short treatment time (about 1 h). Moreover, our findings suggest that the type of stimuli presented during the relaxing and stress phases may modulate both quantitative and qualitative protein composition of saliva.

Discussion

The salivary proteomic analysis was carried out on saliva samples of 36 students divided into two groups which differed for the experimental condition. All subjects underwent a relaxation phase characterized by pleasant auditory stimulation, with or without combination of olfactory stimulation, through the diffusion of a pleasant smell in the room (orange, mint, etc.), while for the subjects belonging to the "auditory stimuli" group the environment remained neutral. All the subjects who entered the study were selected as described in methods, and were subjected during the experimentation to recording of ECG (figure 1) and skin conductance to verify the effective activation of the sympathetic system following the stressful stimulus (Figure 2). It is possible to see how there was a significantly different response of RR and skin conductance in the three phases of the test (rest, study and presentation), showing the progressive increase of heart rate and skin conductance .

Figure 3 shows a representative image of a 2-DE which illustrates the salivary proteomic pattern.

All images of 2-DE gels were analyzed by Progenesis SameSpot. We performed comparative analyses of T1 (exam phase) Vs T0 (relaxation phase) of the two groups.

Proteins found to be differentially expressed after these comparisons and identified with LC MS/MS are shown in panels 1-2.

Four spots, which belong to α-amylase isoforms are increased with a fold change ≈ 1.3 in response to all stress conditions, confirming data reported in literature. The results obtained for immunoglobulins and polymeric immunoglobulin receptor highlighted a decrease of secretion after an acute psychological stress.

Moreover we found a decrease of secretion of many proteins involved in the modulation of immunity response, mainly in the "auditory stimuli" group.

This finding suggests that sensory stimuli (i.e. olfactory) may influence both quantitative and qualitative composition of salivary immuno-related protein components, and can change the physiological response to an acute stress like an oral exam.